Analysis of non-genetic risk factors and *FokI* polymorphism of Vitamin D Receptor Gene in etiology of preeclampsia cases presented at CMH, Muzaffarabad

By

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Department of Zoology
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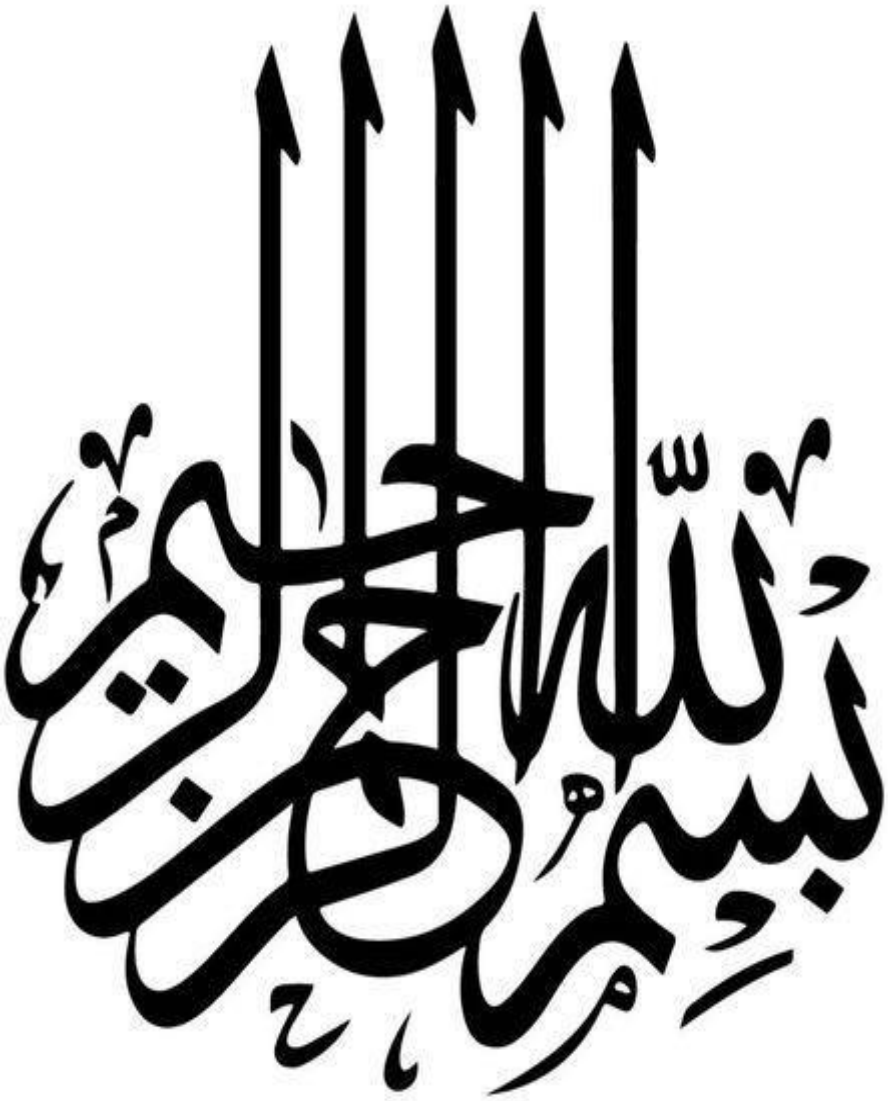
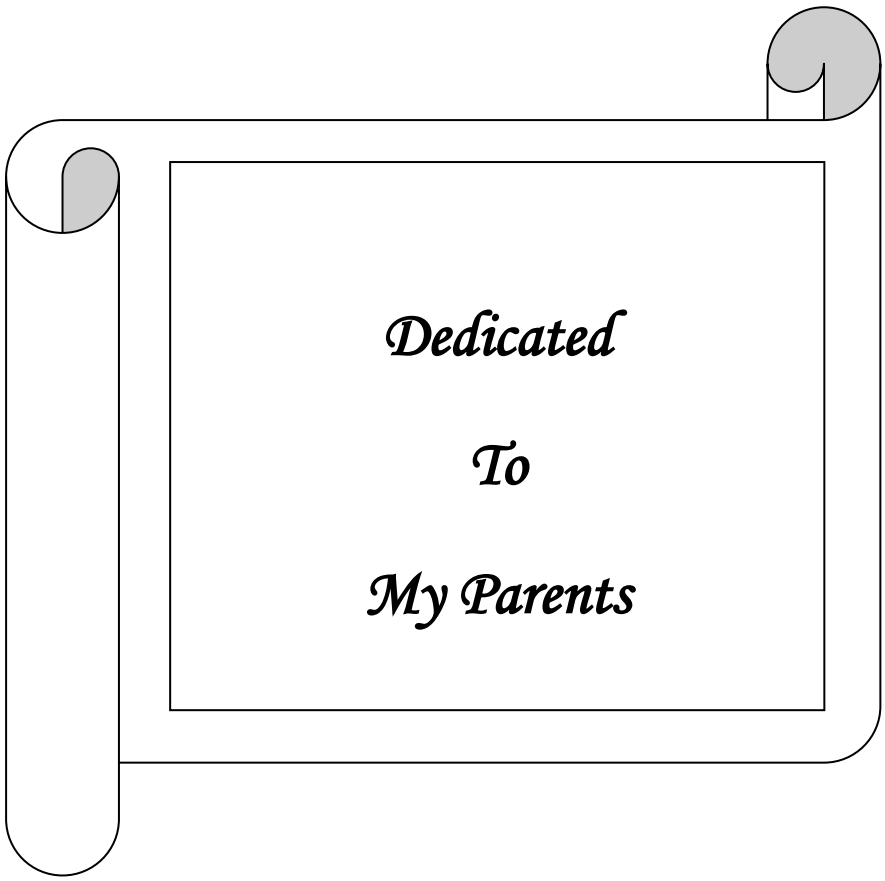
–In the name of ALLAH, the most Beneficent, the most Merciful”




Dedicated
To
My Parents

CERTIFICATE

This thesis **"Analysis of non-genetic risk factors and *FokI* polymorphism of Vitamin D Receptor Gene in etiology of preeclampsia cases presented at CMH, Muzaffarabad"** is submitted by **Iram Shehzadi** is accepted in its present form by the department of Animal sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Zoology.

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DECLARATION

I Iram Shehzadi hereby declare that I have worked on my thesis –Analysis of non-genetic risk factors and *FokI* polymorphism of Vitamin D Receptor Gene in etiology of Preeclampsia cases presented at CMH, Muzaffarabad" independently and the work presented here is original. This thesis has not been submitted in the current or a similar form to any other university.

Iram Shehzadi

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviations	Full form
PE	Preeclampsia
VDR	Vitamin d receptor gene
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate aminotransferase
Hb	Hemoglobin
SDGs	Sustainable development goals
GDM	Gestational diabetes mellitus
PIH	Pregnancy induced hypertension
GH	Gestational hypertension
DM	Diabetes mellitus
ACOG	American college of obstetrics and gynecology
IUGR	Intrauterine growth restriction
NICE	National institute for health and care excellence
BMI	Body mass index
DBD	DNA binding domain
LBD	Ligand binding domain
NK	Natural killer cells
MHC	Major histocompatibility complex
SLE	Systemic lupus erythematosus
VEGF	Vascular endothelial growth factor
sENG	Soluble endoglin
AJK	Azad Jammu Kashmir
AK	Azad Kashmir
UAIs	Unique anonymous identification number
RBCs	Red blood cells
PCI	Phenol chloroform isoamyl alcohol

rpm	Rotation per minute
UV	Ultraviolet
OR	Odd ratio
P-value	Probability value
SD	Standard deviation
N	Number
MTHFR	Methylene-tetra-hydro-folate-reductase
eNOS	Endothelial nitric oxide synthase
CXCR2	Chemokine receptor 2
CYP11B2	Cytochrome p450 family11 subfamily b member 2
TNE	Tris nacl EDTA
SDS	Sodium dodecyl sulfate
TE Buffer	Tris-edta buffer
TBE	Tris/borate/edta
$MgCl_2$	Magnesium chloride
dNTP	Deoxyribonucleotide triphosphate
NaCl	Sodium chloride
EDTA	Ethylene-di-amine tetra-acetic acid
PK	Proteinase kinase
ID	Identification number
CMH	Combined military hospital
QAU	Quaid-i-azam university
DNA	Deoxyribonucleic acid
RDS	Respiratory distress syndrome
HELLP	Hemolysis elevated liver enzymes low platelets counts

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Abstract

Preeclampsia (PE) is a pregnancy specific disorder that results in a maternal and perinatal morbidity as well as mortality. Pregnant women with PE are characterized by endothelial cell dysfunction with many sign and symptoms including maternal organs dysfunction, hypertension, proteinuria and fetal growth restrictions. The etiology of PE still remains unknown therefore, screening tools and preventive measures for PE are lacking. Its treatment involves the management of adverse symptoms in PE patients and early delivery which are the only ultimate cure. Assessments of genetic as well as non-genetic risk factors are considered important for early diagnosis of disease that allows close surveillance and preventive approaches.

For this case-control study, data on demographic characteristics, reproductive factors, medical factors, anthropometric and lifestyle factors was collected from pregnant women presented at CMH hospital Muzaffarabad between April to July 2021. A total of 300 pregnant females, 60 cases and 240 age matched controls were recruited in this study. Demographic characters and PE risk factors were analyzed by cox regression model, while means of the clinical characters of PE patients and controls were compared by independent sample t-test. For molecular analysis, DNA was extracted from blood samples that are collected from PE patients and controls with their consent. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was executed to check the maternal *FokI* rs2228570 polymorphism (T>C) in selected exon 2 of vitamin D receptor (*VDR*) gene. The aim of current study was to investigate the genetic as well as non-genetic risk factors in preeclampsia affected Kashmiri pregnant women.

Results of cox regression model suggested that risk of PE increases significantly ($p<0.05$) with a family history of hypertension and previous miscarriage history (O.R>1, 95%Cl), while multiparity and proper supplements intake during pregnancy were found to be responsible for decreasing the risk of PE (O.R<1, 95%CI). Clinical characteristics i.e. systolic blood pressure (SBP), diastolic blood pressure (DBP), serum creatinine, serum bilirubin, serum urea, alkaline phosphatase (ALP), alanine transaminase (ALT) and urinary protein were significantly elevated ($p<0.0001$) in cases compared to controls, while significantly higher ($p<0.05$) blood haemoglobin (Hb) and platelets count (mean value falls in a normal range) were observed in controls as compared to PE patients. RFLP results of selected exon of *VDR* gene revealed that 20% PE patients were with homozygous CC genotype and 15% with TC genotype while no controls were found with CC or TC

genotypes which indicate that no disease linked variant was present in controls. These results suggest that there is a link between *FokI* rs2228570 polymorphism (T>C) of *VDR* gene and PE risk in our study cohort.

In conclusion some factors such as a family history of hypertension and previous miscarriage history were significantly associated with increased PE risk, while multiparity and proper supplements intake during pregnancy were significantly associated with decreased PE risk. Clinical characteristics such as SBP, DBP, ALP, ALT and urinary proteins were found significantly elevated while blood Hb were significantly lower in PE patients. We identified *VDR FokI* rs2228570 polymorphism in PE patients that suggested the involvement of *VDR* gene in PE pathology in our population. However, future studies with large sample size should be done to further affirm involvement of *FokI* polymorphism of *VDR* gene in PE etiology.

INTRODUCTION

Neonatal and maternal health issues are entirely depends upon the reproductive health (Gunderson *et al.*, 2018). Worldwide community adopted the 17 Sustainable-Development-Goals (SDGs) including improving maternal health. SDG3 addresses, maternal health is the prime priority thereby "ensuring healthy lives as well as well-beings for all at all ages" (Norheim *et al.*, 2015). Since 1990, rate of maternal mortality is reduced to 50 percent. However, the percentage of women who do not survive childbirth is fourteen times greater in developing countries than developed countries (Patton *et al.*, 2009).

Developing countries has absolute maternal fatalities of about 99%, in which South Asia has one third while Sub- Saharan Africa has greater than 50% of it (Sharma *et al.*, 2018). Developing countries has 240 per 100,000 births ratio of maternal mortality, whereas developed countries has 16 per 100,000 (Norheim *et al.*, 2015). Since, countries has significant disparities among regions, so, there can be an exceptional maternal mortality rates of about 1000 per 100,000 live births within some areas (Pasha *et al.*, 2018). In developing countries, both childbirth problems and complicated pregnancies are main cause of neonatal and maternal deaths. As there can be common complications that are related to pregnancies but most of complications are followed by the childbirth and pregnancy. Some appears before gestation which becomes worsen during gestational period (Noh *et al.*, 2019).

Pregnancy associated maternal health complications includes high blood pressure (Agrawal & Wenger, 2020) gestational diabetes (Karasneh *et al.*, 2021), preterm labor (Carter *et al.*, 2018), preeclampsia (PE) (Nirupama *et al.*, 2021) and gestational cholestasis (Luo *et al.*, 2021). All of these have varying severity and maternal as well as fetal outcomes. Some of these pregnancy associated conditions for gestational hypertension, gestational diabetes etc. predispose to future health conditions including cardiovascular disorder (Aslam *et al.*, 2021), type 2 diabetes (Reece, 2010), malignancies, renal failure, etc. (Sheiner, 2020).

1.1 Hypertensive Disorders of Pregnancy

According to the World Health Organization, hypertension is one of the most frequent pregnancy complication in the modern world which complicates about 5–10% of the pregnancies leading to fetal, neonatal and

maternal morbidity and mortality (Regitz-Zagrosek et al., 2018). Many evidences has confirmed that pregnancy-related hypertension may cause early babyhood cardio-metabolic disorder (Davis et al., 2015). The rate of hypertension continues to rise significantly; almost 8% of women with age group between 22–44 years are affected by hypertension in USA (Lu et al., 2018). Hypertension can be defined as a systolic blood pressure (SBP) ≥ 140 mmHg, or diastolic blood pressure (DBP) ≥ 90 mmHg, which should be confirmed with repeated measures after four hours or overnight interval (Beech & Mangos, 2021). According to the severity of blood pressure elevation, pregnancy hypertension is further categorized into severe or mild hypertension.

Severe hypertension: Systolic blood pressure ≥ 160 mmHg and diastolic blood pressure ≥ 110 mmHg.

Mild hypertension: Systolic blood pressure 140–159 mmHg and diastolic blood pressure 90–109 mmHg. Sometimes, it is further categorized into moderate as SBP 150–159/ DBP 100–109 mmHg and mild as SBP 140-149/ DBP 90–99 mmHg (Agrawal & Wenger, 2020).

1.1.1 Categorization

Hypertensive pregnancy disorder is a placental-mediated disorder complicated by many diseases during pregnancy (Hou *et al.*, 2020). American College of Obstetricians and Gynecologists (ACOG) have divided hypertensive pregnancy disorder into following four categories (Obstetricians & Gynecologists, 2013) (Figure 1.1):

- **Pre-existing or chronic hypertension:** Chronic hypertension is high blood pressure before pregnancy or prior to 20 weeks of pregnancy that persists for over 42 days after childbirth.
- **Gestational hypertension (GH**): Gestational hypertension is high blood pressure that develops after 20 weeks of pregnancy and mostly resolves within 42 days after childbirth.
- **Preeclampsia/eclampsia:** Gestational hypertension accompanied by one of the following:
  - Proteinuria
  - Maternal organ dysfunction includes the following:
    - Acute kidney injury,

- Neurological complications (eclampsia, blindness, stroke, severe headache, altered mental status, persistent visual scotomata, clonus)
- Uteroplacental dysfunction (abnormal umbilical artery doppler wave form analysis, fetal growth restriction or stillbirth)
- Liver involvement (transaminitis) with or without epigastric abdominal pain or right upper quadrant
- Hematological complications (disseminated intravascular coagulation, decreased platelet count <150,000/uL, hemolysis)

Gestational hypertension and preeclampsia are the main components of hypertensive pregnancy disorder having worldwide incidence of about 0.2–9.2% and 1.8–4.4% respectively, but their incidences have seasonal and regional differences (Morikawa *et al.*, 2014; Umesawa & Kobashi, 2017).

❖ **Pre-existing/chronic hypertension with superimposed eclampsia-preeclampsia**

A woman with chronic hypertension shows symptoms of eclampsia or preeclampsia after 20 weeks of gestation. When high blood pressure is developing after 20 weeks of pregnancy that usually resolves within 42 days after childbirth, the European Society of Cardiology (ESC) has categorized it into antenatally unclassifiable hypertension (Regitz-Zagrosek *et al.*, 2018).

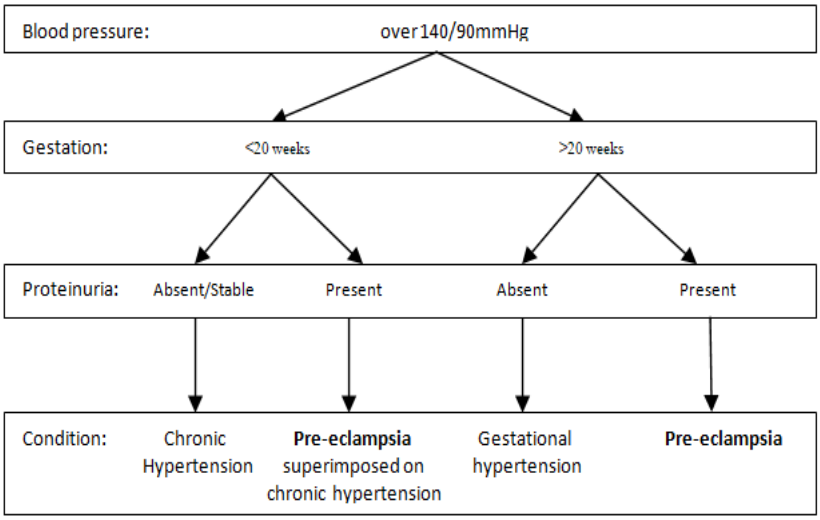

Figure 1.1 Diagnostic criteria to differentiate various types of hypertension and PE (Adopted from Portelli & Baron, 2018)

1.2 Preeclampsia

Preeclampsia is the pregnancy specific disorder and it affects about 3–5% of the pregnancies arising from the abnormal placentation that results in oxidative stress, an

imbalance of angiogenic and anti-angiogenic factors, and immunological involvement. It usually occurs after 20 weeks of gestation and is diagnosed when a pregnant women has proteinuria and hypertension (Beech & Mangos, 2021; Mol *et al.*, 2016). Approximately 10% of the pregnant women have their blood pressure above the normal level during pregnancy. Pre-eclampsia, is now known as a complex multisystem progressive disorder of pregnancy that complicates about 2-8% of pregnancies in a Western world and 5% of the primiparity (North *et al.*, 2011). Preeclampsia and eclampsia are responsible for approximately a third of a million deaths in low and middle income families and more than six million perinatal deaths (Bilano *et al.*, 2014). According to a systemic analysis of global mortality, Pakistan has the third-highest burden of fetal, child, and maternal mortality (Bhutta *et al.*, 2013).

Protein loss of ≥300 mg in 24-hour urine specimen collection should be defined as proteinuria. For diagnosis of preeclampsia, proteinuria/creatininuria ratio ≥0.3 mg/dL in urine sample should be considered adequate.

In the absence of these diagnostic possibilities, preeclampsia can be diagnosed by the presence of visual turbidity, headache, abdominal pain or altered laboratory tests, such as hepatic enzyme elevation (double the basaline), thrombocytopenia (<100,000/mm3), pulmonary edema, renal impairment (more than 1.1 mg/dL or double the baseline), and visual or brain syndrome such as headache, convulsions or scotomas (Ramos *et al.*, 2017).

Glomerular damage is caused by proteinuria. On urine dipstick analysis, if +1 protein value is greater than 300mg in urine sample collected in a 24 hour, or value of 0.3 mg/dL or more of protein/creatinine ratio is known as preeclampsia (Obstetricians *et al.*, 2013; Okten *et al.*, 2018).

1.2.1 Clinical definition of preeclampsia

Boissier de Sauvages discriminated eclampsia from the epilepsy in 18th century (Temkin, 1994). Eclamptic hypertension was discovered by Vaquez and Nobecourt (Chesley, 1978). After this discovery, the concept of PE was documented. It was then considered that the presence of proteinuria, headaches and edema might have role to cause convulsions (Sinclair, 1858).

According to the American College of Obstetrics and Gynecology (ACOG), preeclampsia can be defined as the normotensive patient presented with high blood pressure along with proteinuria which occurs after 20 weeks of pregnancy. The delayed in

diagnosis occur when a greater proportion of women show symptoms like low platelets, renal insufficiency or elevated liver enzyme before the detection of proteinuria. The ACOG's hypertension 2013 task is forced by the better understanding of preeclampsia as a hypertensive pregnancy disorder to revise the definition of preeclampsia by including the presence of harsh features even with or without proteinuria and also excluding the level of proteinuria as a criterion of harsh features (Figure 1.2) (Obstetricians & Gynecologists, 2013). In a recently updated ACOG's practice guidelines, these criteria were confirmed (Obstetricians & Gynecologists, 2019; Rana *et al.*, 2019).

Preeclampsia
Elevated blood pressure
Systolic ≥140 mm Hg or diastolic ≥90 mm Hg, 2 occasions, 4 h apart in previously normotensive woman
AND Proteinuria
≥300 mg/24 hour urine collection
or protein/creatinine ≥0.3
or dipstick reading =1+
OR severe features*
Severe features
Systolic blood pressure ≥160 mm Hg or diastolic ≥110 mm Hg, 2 occasions, 4 h apart on bedrest
Thrombocytopenia (<100 000 μL)
Liver function tests 2× normal or severe persistent right upper quadrant or epigastric pain
Serum creatinine concentration >1.1 mg/dL or doubling of creatinine in the absence of other renal disease
Pulmonary edema
New-onset cerebral or visual symptoms

Figure 1.2 Clinical definition of preeclampsia (Adopted from Obstetricians & Gynecologists, 2013)

1.2.2 Sub-classification

1.2.2.1 Early and late onset preeclampsia

Further query arises on the "early and late onset" variants of preeclampsia whether they are same or has a completely separate pathological mechanism of disease. Another point of discussion is how to define the "severe and mild preeclampsia" (Smith *et al.*, 2001).

Preeclampsia is mostly defined as the early onset (preeclampsia when diagnosed before 34 weeks of gestation) or late onset (diagnosis occur ≥34 weeks of gestation), where patient with early onset preeclampsia is often associated with severe placental dysfunction

and intrauterine growth restrictions (IUGR) of fetus (Tranquilli *et al.*, 2014). A small portion of preeclampsia cases (5 to 20 percent) relates to early onset of preeclampsia, though it is clinically more important (Parra-Cordero *et al.*, 2013). The variations of blood flow in placental uterine and spiral arteries, an insufficient trophoblast invasion of the maternal spiral arteries are the features of early onset preeclampsia. Fetal growth restriction and high resistance in peripheral area of placental vessels are the main basis for abnormal blood flow in the umbilical arteries (Huppertz, 2008).

From all preeclampsia cases, about 80 percent cases are linked to late onset preeclampsia. A large portion of the early onset of preeclampsia are associated with normally developed infant without any sign of growth restrictions, no changes occur in the blood flow of umbilical arteries but there is a moderate or an ordinary adjustment occurs in the behavior of uterine spiral arteries and there is more danger for pregnant women with diabetes, multigravida, increased mass of placenta, high elevation etc. (Myatt & Roberts, 2015).

1.2.2.2 Severe and mild preeclampsia

Depending on the intensity of sign and symptoms, preeclampsia can be classified as severe preeclampsia, mild preeclampsia and eclampsia (Grill *et al.*, 2009). Preeclampsia is considered as mild when systolic blood pressure is 140 mmHg and diastolic blood pressure is 90 mmHg, the proteinuria level is ≥300 mg in 24hour urine sample, and the proteinuria/creatininuria ratio ≥0.3 or 30 mg/dL in urine sample (on dipstick 1+ reaction). While severe preeclampsia is characterized by having systolic and diastolic blood pressure of 160mmHg/110mmHg respectively, the proteinuria/creatininuria ratio ≥3+, impaired liver function in association with visual abnormality, pneumonic oedema, thrombocytopenia, oligouria (under 500 mL for every 24-hour time frame), elevation in the serum level of creatinine and transaminase, seizures, upper abdominal or epigastric pain or cerebral pain. Presence of seizures is the main characteristic for eclampsia.

1.3 Risk Factors

According to guidelines of National Institute for Health and Care Excellence (NICE) 2019 (Alliance, 2019) that pregnant women were found to have previous record of hypertensive disorder or maternal diseases viz chronic hypertension, autoimmune diseases, renal disease, or diabetes would results into higher risk of preeclampsia. Women having body mass index (BMI ≥ 35 kg/m), multigravidity, family record of PE, nulliparity, >10

years pregnancy interval, age ≥40 years were reported to have a moderate risk (Alliance, 2019; Brown *et al.*, 2018). These risk factors are investigated by the Bartsch *et al.* (Bartsch *et al.*, 2016), in the meta-analysis of more than twenty five million pregnancies conducted through several researchers in 92 studies for medical risk factors. Women with ≥2 moderate risk factors or one high risk factor should guide for aspirin prophylaxis, an effective drug to reduce the risk of PE if taken before 16th gestational week (Askie *et al.*, 2007; Bujold *et al.*, 2010).

Additionally, there are some risk factors that relatively enhance the clinical signs of preeclampsia that includes polycystic ovarian disorder (Bahri Khomami *et al.*, 2019; Qin *et al.*, 2013; Yu *et al.*, 2016), sleep breathing syndrome (Pamidi *et al.*, 2014), elevation of blood pressure in arteries before 15th gestational week (North *et al.*, 2011), at least 5 days vaginal bleeding in obstetric history (North *et al.*, 2011), and many infections like urinary tract infection, helicobacter pylori and periodontal disease (Bellos *et al.*, 2018; Rustveld *et al.*, 2008). There is also a high risk of PE in using donated oocyte in invitro fertilization as compared to the natural conception (Blázquez *et al.*, 2016; Jeve *et al.*, 2016; Masoudian *et al.*, 2016). Many studies have investigated that vitamin D deficiency could be a risk factor for PE that can be reduced by taking vitamin D supplements (Akbari *et al.*, 2018; Ali *et al.*, 2019; Arain *et al.*, 2015; Mirzakhani *et al.*, 2016; Nassar *et al.*, 2011; Purswani *et al.*, 2017; Wei *et al.*, 2013).

1.4 Genetic Influences

There are numerous genetic factors that are involved in the development of PE. Because of the complicated pathophysiology of preeclampsia, it is considered that many genes play a part for the development of preeclampsia. Majority of the researchers paying attention on the mutation and polymorphism of susceptible maternal genes, including endothelial nitric oxide synthase gene (Salimi et al., 2012), methylenete-trahydrofolate reductase gene (Salimi, Farajian-Mashhadi, et al., 2015), Vitamin D receptor gene (Purswani et al., 2017) and Angiotensin-converting enzyme (Salimi et al., 2011).

1.4.1 Role of vitamin D receptor (*VDR*) gene in preeclampsia

Studies have reported that vitamin D deficiency can cause preeclampsia (Bodnar *et al.*, 2007). Several observational studies have suggested that pregnant women with low vitamin D level are at greater risk of preeclampsia (Fischer *et al.*, 2021; Robinson *et al.*, 2010). Numerous biological processes that are linked to the pathogenesis of preeclampsia including hypertension, angiogenesis, placenta implantation and immune dysfunction

could be affected by the vitamin D level (Evans *et al.*, 2004; Hewison, 2012; Li *et al.*, 2002).

As the active form of vitamin D is mainly produced in placenta, immune modulation in placenta and calcium transfer into placenta is influenced by the vitamin D that are necessary for the proper maternal immune response to maintain the innate immunity as well as favors placenta implantation. Studies have reported that endothelial vitamin D receptor (*VDR*) expression is reduced by the low vitamin D level (Murthi *et al.*, 2016). To perform the biological functions of vitamin D, it is important that active form of vitamin D [1, 25 (OH)2 D] bind to the vitamin D receptor (*VDR*). Vitamin D receptor (*VDR*) gene belongs to a superfamily of the nuclear receptors for steroid hormones and is localized on the chromosome 12. *VDR* gene contains 2 globular domains, one is DNA-binding domain (DBD) and the other is ligand-binding domain (LBD) and it also regulates some specific genes involved in lipid metabolism, glucose modulation as well as blood pressure (Baker *et al.*, 1988; Labuda *et al.*, 1992).

One or many signaling cascades regulating immune function, calcium metabolism, cell proliferation and differentiation get activated by the binding of the active form of vitamin D to *VDR* (Bikle, 2009; Samuel & Sitrin, 2008). During pregnancy, *VDR* gene expression, which is an important stage in the vitamin D metabolic pathway in the placenta, is finely changed (Shahbazi *et al.*, 2011). *VDR* expression has been found to be much higher in the syncytiotrophoblast and cytotrophoblast during pregnancy, and it is also enhanced in the deciduous and placenta as compared to endometrium (Fischer *et al.*, 2021; Pospechova *et al.*, 2009). Research suggested that there is a substantial link between *VDR FokI* and *BsmI* variants that might have effect on vitamin D binding and risk of developing hypertension (Wang *et al.*, 2013). Furthermore, multiple sclerosis (Narooie-Nejad *et al.*, 2015), cancer (Gandini *et al.*, 2014) and pulmonary tuberculosis (Salimi, Saravani, *et al.*, 2015) have all been linked to *VDR* gene polymorphisms. We hypothesized that *VDR* could be involved in the development of PE because of its crucial role during pregnancy and large alterations in *VDR* expression in PE. As a result, the current study looked into the possible link between placental and maternal polymorphisms of *VDR* gene and PE risk.

1.4.1.1 *FokI* polymorphism and risk of preeclampsia

A polymorphism is an inherent variant and it appears in more than one percent of population. *VDR* gene is extremely polymorphic in nature. Among different populations,

the frequencies of alleles of *VDR* gene are inconsistent which suggested that its polymorphism might affect the activity, stability and quantity of *VDR* protein (Zhan *et al.*, 2015). *VDR* gene *FokI*, *BsmI*, *TaqI* and *ApaI* polymorphisms are reported to be linked with increased risk of PE (Farajian-Mashhadi *et al.*, 2020; Rezavand *et al.*, 2019). *VDR* gene polymorphism in 5ˋ- promoter sequence can influence the protein translation efficiency and mRNA expression patterns as well as levels, while in 3ˋ – untranslated region (UTR) can affect the protein translation efficiency and stability of mRNA. Few variants have been studied but their functional effect is unknown that makes difficult to interpret the variants of *VDR* gene. Therefore, it is necessary to know how different polymorphisms of this gene relate with each other at functional as well as genetic level (Singla, 2015).

The *FokI* polymorphism involves substitution of T (thymine) by C (cytosine) in many translation initiation codon i.e. methionine (ATG), therefore it is also known as start codon polymorphism (SCP). The translation initiation at first start codon resulted in full length *VDR* protein of 427 amino acids. However, when translation initiates at second start codon, it produces a truncated protein with a reduction of three amino acids but with greater biological activity, which is thought to be linked with reduced risk of hypertension (Singla, 2015). Several studies reported that *FokI* polymorphism rs2228570 (c.2T>C) of *VDR* gene is associated with an increased risk of PE (Rezavand *et al.*, 2019; Zhan *et al.*, 2015).

1.5 Epidemiology

In developing countries, PE is a serious pregnancy complication which later became a reason of maternal mortality, PE along with seizures leads to eclampsia which account for more than 10% of maternal mortality. 15-20% maternal mortality is caused by PE in developed countries. PE is also a major cause of perinatal deaths, intrauterine growth restrictions, maternal morbidity, and preterm birth. The estimated rate of occurrence of PE is 1 in 20 pregnancies. From PE, annually about 50,000 worldwide maternal deaths occur. According to definition of PE and the studied population, about 2-10% is the occurrence rate of PE and it complicates about 5-8% of worldwide pregnancies which leads to many serious complications in both child and mother (Shamsi *et al.*, 2013; Sibai *et al.*, 2005).

World Health Organization stated that pregnancy hypertensive disorders leads to 26 percent maternal mortality in the Caribbean and Latin America, 9 percent maternal mortality in Asia and Africa, and approximately 16 percent maternal mortality in the developed countries (Khan *et al.*, 2006).

The prevalence of PE differ in different countries viz 12 percent in the Bangladesh (Present, 2015), 8.9 percent in Brazil (Zeiger *et al.*, 2015), 4.7 percent in Thailand (Pitakkarnkul *et al.*, 2011), 3.4 percent in United States (Ananth *et al.*, 2013), 3.3 percent in Australia (Thornton *et al.*, 2013), and 3.2 percent in India (Perveen, 2014). Most of maternal mortality is caused by eclampsia instead of preeclampsia, but the prevalence of eclampsia is less than preeclampsia upon introducing prediction methods and management of preeclampsia (Duley, 2009; Hutcheon *et al.*, 2011; Jeyabalan, 2013). In developing countries 15% maternal deaths are attributable to eclampsia, while currently it is rarely found in developed countries (Ghulmiyyah & Sibai, 2012). More ICU admissions, stillbirths and cesarean section are attributable to eclampsia in comparison to preeclampsia. Low birth weight and fetal respiratory distress is caused by eclampsia (Knight, 2007; Lal *et al.*, 2013). The prevalence of eclampsia was found to have 1.03% in India (Kharaghani *et al.*, 2016; Perveen, 2014), 0.08% in the United States (Lal *et al.*, 2013), 0.03% in Qatar (Sharara, 2013), and 0.02% in the United kingdom (Knight, 2007).

The prevalence of preeclampsia and eclampsia was about 19 percent in Pakistan where 1 in 89 pregnant women dies of the maternal reason (Naseeb *et al.*, 2015; Shamsi *et al.*, 2010). Annually about 500,000 fetal or infant and 76,000 worldwide maternal fatalities are caused by preeclampsia (Carty *et al.*, 2010; Duley, 2009; Khowaja *et al.*, 2016).

1.5.1 Fetal and neonatal effects

PE related fetal and infant outcomes vary around different regions of world. Approximately 15 to 20 percent of all premature births and 12 to 25 percent of intrauterine growth restriction are caused by PE. There can be extensive complications regarding prematurity/preterm birth which eventually leads to neonatal morbidity and mortality (Duley, 2009). In developing countries, ¼ of all stillbirths and infant mortality are attributable to preeclampsia/eclampsia. Due to insufficient facilities for neonatal care, preeclampsia related neonatal mortality is three fold higher in developing countries than developed countries (Duley, 2009; Jeyabalan, 2013).

1.6 Clinical Presentation

Women with preeclampsia clinically present visual disturbance, headache, upper abdominal pain and seizures in severe cases (Young & Karumanchi, 2016). Preeclampsia can be predicted by hypertension which is the first sign but sometimes it does not appear until the late phase of disease, and complete obstetric and neurological

examination can be done to diagnose the disorder (August & Sibai, 2017; Mol *et al.*, 2016). Proteinuria is a major clinical sign for early diagnosis in which urinary protein are examined (Myatt & Roberts, 2015). Preeclampsia is characterized by quickly progressive hand and face oedema, however feet oedema is also common in normal pregnancy (Figure 1.3) (Portelli & Baron, 2018). Epigastric pain is the alarming sign and it is thought that liver is involved in it (Steegers *et al.*, 2010). In severe disease, neurological examination also reveals hyperflexia and clonus. Preeclampsia is a heterogenous syndrome which results in multiple maternal organ dysfunctions (Eiland *et al.*, 2012). Extremely variable severity of the clinical signs and symptoms of preeclampsia is present, mild preeclampsia occur after 36th gestational weeks results in a favourable outcomes. When preeclampsia builds up before 33 weeks of gestation, there may be a risk of unfavourable maternal and infant outcomes (Duckitt & Harrington, 2005).

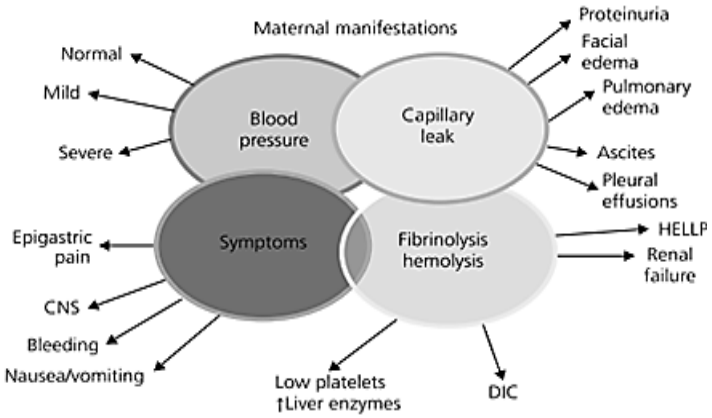

Figure 1.3 Organ dysfunction and symptoms of PE

1.7 Patient Evaluation

Preeclampsia should be predicted by new onset hypertension or severe hypertension in a normotensive pregnant woman after 20 weeks of pregnancy. Pregnant women with severe hypertension and symptoms such as epigastric pain, cerebral impairment, dyspnea, or visual symptoms is an indication for severe PE, which must be hospitalized to evaluate the initial clinical features of mother and fetus and to manage it accordingly (August & Sibai, 2017).

- **Accurate assessment of blood pressure**
- **Laboratory tests** — to evaluate the PE, following laboratory test should be examine:

- ✓ Liver chemistries (alanine aminotransferase [ALT] and aspartate aminotransferase [AST],)
- ✓ Complete blood count with platelets
- ✓ Proteinuria level (protein and creatinine ratio in a 24-hour urine sample or a random urine sample collection)
- ✓ Serum creatinine level (August & Sibai, 2017)

1.8 Etiology and Pathophysiology

Although the etiology of preeclampsia is still not clear, the pathogenesis is thought to happen in two major phases. The first phase is characterized by an abnormal placentation, and the second phase begins by the abnormal maternal endothelial response which consequently leads to hypertension, oedema, and proteinuria (Carty, 2012).

1.8.1 Placental phase

In the development of PE, placenta plays a major role. It is known that PE is the pregnancy specific disorder that ultimately resolves after the placental delivery, and it may happen in the absence of viable fetus such as in the molar pregnancies. Placentation is tightly regulated process which is necessary for the development of normal fetus, where spiral and uterine arteries supply blood to placenta (Carty, 2012).

In some stages of pregnancy, remodeling of spiral arteries occur that begins at the time of implantation. This remodeling is necessary for the normal development of placenta, which alters the arteries from a low-flow and extremely resistant vessels into a high-flow and low resistant vessels (Figure 1.4). In the pathogenesis of PE, false remodeling of spiral arteries is considered as an important factor (Steegers *et al.*, 2010).

There is impaired remodeling of the spiral arteries occurs at the time of implantation in preeclampsia, which provides an explanation of the fact that pregnant women with previous miscarriage or a history of sub-fertility are at high risk of this condition (Steegers *et al.*, 2010).

Intervillous flow begins at 7-8 weeks of gestation which is characterized when the connecting channels appears between the blastocyst and the spiral arteries. The developing placenta‘s cytotrophoblastic cells invades into the decidual segments of spiral arteries at about 10-12 gestational weeks and then it invades into the myometrial segments at around 15-16 gestational weeks. The trophoblast formed then invades into both the higher muscular tunica media of maternal spiral arteries and endothelium (Carty, 2012).

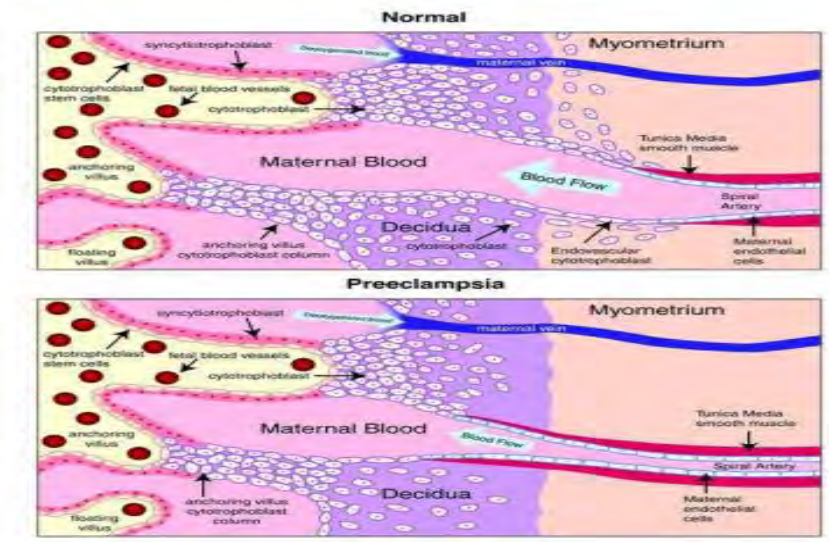

Figure 1.4 Cytotrophoblasts invading the maternal spiral arteries transforming them from high-flow, low-resistance vessels in normal pregnancy (top) and impaired remodeling in pre-eclampsia (bottom) (Adapted from Powe *et al.*, 2011)

The Pre-eclampsia involves the invasion of cytotrophoblast into the decidual portion of the spiral arteries but abnormal invasion of myometrial segments, the spiral arteries becomes narrow resulting in the restriction of blood supply to the foetus. These effects becomes more significant on the foetus as the pregnancy progresses since the uterine vasculature do not receive the sufficient amount of nutrients and blood needed for the foetal development (Carty, 2012).

The cause of the impaired development of the uteroplacental circulation in PE is unknown and is a matter of debate. Vascular, genetic and environmental factors are thought to play a key role (Ilekis *et al.*, 2007). In these early stages of PE development, the maternal natural killer (NK) cells are thought to have a key role. Natural killer cells, the main maternal immune cells in endometrium before implantation plays a key role to regulate the development of placenta. The interaction of maternal natural killer cells with fetal major histocompatibility complex (MHC) antigens may represent the initial step. The combinations of fetal MHC-C and maternal NK cells genotype are linked to impaired development of placenta and its association with high risk of PE and miscarriage has been reported (Colucci *et al.*, 2011). Ongoing research on the interaction between foetal gene expression and natural killer cells may help in gaining the better understanding of the vital early stages in development of PE.

Hypoperfusion of the placenta from impaired remodeling of the spiral arteries may results in impaired development of placenta. Accordingly, conditions related

with vascular insufficiency, including systemic lupus erythematosis (SLE), hypertension, renal disease and diabetes can increase the risk of development of PE and abnormal placentation (Duckitt & Harrington, 2005). Placental ischaemia is caused by hypoperfusion of the developing placenta; pathological sign that indicate ischaemia include thrombosis, atherosis, placental infarction and fibrinoid necrosis. Although not all women with pre-eclampsia have the typical placental pathology features, their presence appears to be linked to severity of disease (Salafia *et al.*, 1998). When the hypo-perfused, ischemic placenta releases a variety of substances in maternal circulation, the interface between the maternal and placental components of PE development is hypothesized to occur (Lee *et al.*, 2007).

1.8.2 Maternal response

In a comparison to ordinary pregnancy, the exaggerated pro-inflammatory condition as well as activation of maternal endothelial plays a key role in the second phase for development of PE (Redman & Sargent, 2010). Oxidative stress, discharge of components from intervillous space into maternal circulation, and syncitial architecture damaging occur in a result of placental hypoxia. In a maternal circulation, the remains of trophoblast includes microparticles of syncytiotrophoblastic membrane, soluble type of the vascular endothelial growth factor (VEGF) receptor i.e (sFLT-1), and the factors generated by the syncytiotrophoblast as well as the soluble endoglin (sENG). These and some other unidentified factors causes increased vascular reactivity, endothelial dysfunction and release of inflammatory cytokines in maternal circulation. As a result, maternal endothelial integrity is loosed that leads to abnormal physiological vascular alterations during pregnancy. Consequently, oedema, hypertension and proteinuria are caused (Figure 1.5) (Parikh & Karumanchi, 2008).

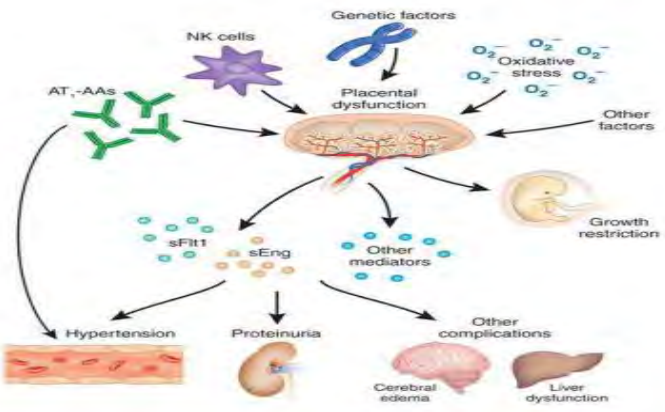

Figure 1.5 Diagrammatic presentation of pathophysiology of PE (Adapted from Parikh & Karumanchi, 2008)

1.9 Management of Preeclampsia

The only evidence based treatments for lowering the incidence of preeclampsia (primary intervention) are aspirin for high risk women (only lowering the incidence of preterm preeclampsia) and calcium supplement, especially in certain populations with low calcium intake.

The only definite treatment for PE is the delivery of placenta before 34th gestational week to avoid serious maternal problems, but it can also cause severe neonatal damage (Lisonkova & Joseph, 2013; Pennington *et al.*, 2012). To overcome the disease, sufficient prenatal care is needed. For the management of PE, assessment of disease, severity review and admission to the hospital should be considered. The high risk of PE in pregnant women can be reduced by adopting some strategies like standard assessment and examining the disease, prevention of high blood pressure by taking hypertensive drugs such as Labetalol (an alpha and beta blocker and Nifedipine that blocks the calcium channel) (Duro-Gómez *et al.*, 2017), avoidance of severe rehydration (Steegers *et al.*, 2010), treatment of seizures by taking medicines if needed which can be a source of magnesium sulphate to reduce the fits (Bachnas *et al.*, 2021; Ueda *et al.*, 2016).

Women with high risk of PE needs more care with increased antenatal observation while low risk women will be allowed to take part in a community-based antenatal care (Ukah *et al.*, 2018). Additionally, some novel therapeutic preventive measures can be formed through prognostic tests. Unfortunately, neither a screening test for PE nor a single blood biomarker identified having sufficient specificity and sensitivity to clinically assist in management of PE either alone or together with any other biomarker, instead of intensive research on PE (Magee *et al.*, 2014; Wu *et al.*, 2015).

Impaired trophoblastic remodeling of the spiral and uterine artery examined by doppler ultrasound assists in finding the risk of preeclampsia. This kind of screening test is valuable for pregnant women with high risk of PE recognized by the previous medical and post obstetric history (Khong *et al.*, 2015). Though, it is not helpful for screening women with low risk of disease.

The only treatment and preventive measures to reduce the incidence of PE (primary intervention) includes low dose aspirin that reduces the risk of preeclampsia in high risk women and calcium supplement may also reduce the risk in certain women with low calcium intake. Studies reported that vitamin C or E cannot decrease the risk of preeclampsia (Mohammad-Alizadeh-Charandabi *et al.*, 2015; Szymański *et al.*, 2015).

In case of severe preeclampsia, premature fetal delivery by the cesarean section is required. After placental delivery, both hypertension and proteinuria will resolve, but in few cases, they will persist more than 6 weeks if a woman has chronic hypertension or any renal disease (Magee *et al.*, 2014). Additionally, women having severe preeclampsia before 34th gestational week should be considered for intensive post natal research (Nathan *et al.*, 2018). To avoid neonatal and maternal mortality as well as morbidity; a research is needed to find the actual cause and process that was involved in pathogenesis of preeclampsia.

Referred to the above reviewed literature, present study was designated to evaluate the demographic as well as genetic risk factors in the development of preeclampsia in pregnant women presented at CMH hospital between four months duration of April 2021 to July 2021. By keeping in view the role of *VDR*-gene *FokI* polymorphism was selected to be studied using restriction fragment length polymorphism (RFLP).

1.10 Aim of Study

Since preeclampsia is poorly understood hypertensive pregnancy disease, therefore there is no specific standard for prognosis of preeclampsia. Researchers continuously searched for diagnostic parameters in order to forecast the severity and progression of disorder. Several epidemiological, genetic and clinical risk factors should be checked before it develops threat to both fetal survival and mother. Azad Kashmir is economically developing state of Pakistan; several studies have been reported about PE from different areas of Pakistan (Arain *et al.*, 2015; Ejaz *et al.*, 2020; Hoodbhoy *et al.*, 2021; Salam *et al.*, 2015; Shamsi *et al.*, 2010), but there is not any single study in Azad Kashmir have been reported till now. Therefore, it is addressed in current study. It is highly needed to identify the women which are prone to higher PE risks so that they can benefit from it. Therefore, current study was carried out for the assessment of the predictive role of clinical, demographic and molecular risk factor of PE in Kashmiri women.

1.11 Objectives

Present study was planned with following main objectives:

- To evaluate clinical and demographic risk factor of PE in Kashmiri women.
- To screen *FokI* polymorphism rs2228570 of *VDR* gene by RFLP in normal healthy as well as preeclampsia patient.

MATERIALS AND METHODS

Epidemiological and molecular study of preeclampsia (PE) involved following steps:

1. **Field work**
   - Approval was taken from CMH hospital to enroll patients by signing informed consent.
   - A predesigned questionnaire was used to record demographic and clinical risk factors of PE for all patients and controls.
2. **Statistical analysis of genetic and non-genetic risk factors**
3. **To study *FokI* polymorphism, following steps were involved:**
   - Reagent preparation for DNA extraction
   - DNA extraction
   - DNA quantification through nanodrop
   - Primer designing
   - Preparation of working dilutions of primers
   - Polymerase chain reaction (PCR)
   - Horizontal gel electrophoresis
   - Restriction fragment length polymorphism (RFLP)
   - Horizontal gel electrophoresis

Section A-Field Work

2.1 Approval from hospital

The study was approved by the Supervisory Committee of Quaid-i-Azam University Islamabad Department of Zoology. Then permission was taken from the Department of Medical Education of Combined Military Hospital (CMH) Muzaffarabad, AJK. Sampling was done from Gynae Department of CMH Muzaffarabad. The study included all the preeclampsia patients who visited the hospital between April 2021 and July 2021, independent of family history of disease, age, clinical presentation or other factors.

2.2 Participants

During the period of April to July 2021, total 300 pregnant women were recruited in this study, from which 60 were those who suffers from preeclampsia while 240 were healthy normal controls. A detailed questionnaire including clinical examination, a history

of disease and demographic data as well as an informed consent were taken before the collection of sample.

2.3 Inclusion and exclusion criteria

The inclusion criteria were: age ≥17 years and delivery within a period of April to July 2021. Diagnosed patients with preeclampsia having systolic and diastolic blood pressure of ≥140/90 respectively along with proteinuria ≥300 mg per day or ≥1+ on dipstick calculated on two different occasions at least 6 hours apart were included in this study. While the non-preeclamptic women were taken as a control for this study having blood pressure ≤125/85 as well as no proteinuria. Individuals having abnormal embryo seen in ultrasound, severe kidney disorder, malignant syndrome and consumption of immunosuppression and anticancer treatment were excluded.

2.4 Blood Sampling and Storage

About 3µL of blood sample were collected in an EDTA (ethylene diamine tetra acetic acid) tube which is already labeled with specific ID number. Tubes were inverted for 5 to 6 times to thoroughly mix the sample with EDTA present in tube and stored in a freezer at -80 °C till further analysis.

2.5 Questionnaire

After the patient's assent, the questionnaire was filled by interviewing patients about their lifestyle factors, family history of disease as well as medical history. Informed consent that was used is attached as an annexure I.

2.6 Exposure Assessment-Characterization of Established and Suspected Risk Factors

An age matched case control study was conducted. The supervisory committee of Quaid-i-Azam University Islamabad had previously approved the study, and the patients had given their informed consent prior to participation. 300 patients matched the inclusion criteria. At enrolment, biophysical variables i.e. systolic and diastolic blood pressure, blood hemoglobin, body mass index, infant weight and the personal and family history of patient i.e. age, age at marriage, educational status, profession, smoking status, consanguinity, parity, previous PIH, chronic hypertension, diabetes mellitus (DM), gestational diabetes mellitus (GDM) and family history of PIH in her mother were recorded. Electronic sphygmomanometer devices authorized by the Chilean Cardiologic Society were used to

measure systolic and diastolic blood pressure. BMI was calculated by using the formula weight / (height*height)

2.7 Selection of Patients for Genetic Studies

All enrolled patients of PE were selected for the genetic analysis.

Section B-Compilation of Patients Data and Risk Factor Analysis

Microsoft Excel 365 was used to compile all the data collected for different variables. Different computational tools were used to analyze genetic as well as environmental risk factors. The control and patient data were used in a cox regression analysis using IBM SPSS statistics version 20 software to determine which risk factors are responsible for the progression of preeclampsia in the study area.

Section C-Lab Work at Molecular Biology Lab For Molecular Analysis:

- Reagent preparation for DNA extraction
- DNA extraction
- DNA quantification through nanodrop
- Designing of primer through computational software
- Preparation of working dilutions of primers
- Polymerase chain reaction (PCR)
- Agarose gel electrophoresis
- Restriction fragment length polymorphism (RFLP)
- Agarose gel electrophoresis

2.8 Extraction of genomic DNA

The patients' peripheral blood approximately 3ml was collected in EDTA k3 vacutainers, which already contained EDTA to prevent clotting of blood. It was appropriately labeled with Unique Anonymous Identification Numbers (UAIs) assigned to each patient in order to keep them different. The tubes were inverted three to four times to mix the EDTA which is already present in the tube with the blood. Blood samples were then placed in the freezer at -20°C until the DNA was extracted by using modified phenol chloroform method which is as follows:

1st Day of DNA extraction

- EDTA tubes containing blood samples were thawed that causes the lysis of Red Blood Cells (RBCs) at room temperature.

- Patient UAI numbers were labeled on the 50 ml falcon tubes.
- Blood was transferred from EDTA tubes into the already labeled falcon tubes.
- Recorded the volume of blood in each tube.
- Washing TE buffer was prepared by adding 4 mL EDTA and 10 mL Tris, then filled it upto rim of bottle with Autoclaved filtered water and mixed thoroughly.
- Added washing TE buffer up to 40ml to each falcon tube.
- Centrifuged the tubes at 3000 rpm and 20 °C temperature for 20 minutes.
- Two layers formed after centrifugation. The upper layer is known as supernatant, while the lower layer is known as the pellet.
- Using autoclaved glass pipettes, up to 20 ml of supernatant was discarded from each tube.
- Each tube was refilled up to 40 ml with washing TE buffer, and then pellet was dissolved by tapping.
- Again centrifuged the tubes at 3000 rpm and 20 °C for 20 minutes.
- Now up to 10 ml of supernatant was discarded and again TE buffer was poured up to 40ml. After dissolving the pellet, centrifuged it at the same conditions.
- Discarded all the supernatant after third washing, leaving the pellet in the tube.
- Then, in each falcon tube, calculated amount of 20 percent SDS, proteinase kinase (PK) and TNE buffer was poured according to the volume of blood, mixed well and placed it in the incubator overnight at 37 ºC temperature.

2nd Day of DNA extraction

- The Falcon tubes were removed from the incubator and were placed at temperature.
- 500µl of sodium chloride (NaCl) was poured into each tube. After vigorous shaking the tubes were placed on the crushed ice.
- Prepared the phenol, chloroform, and isoamyl alcohol mixture (PCI) and then 1mL PCI was poured into each tube.
- Tubes were then centrifuged at 3000 rpm and 20°C temperature for 20 minutes. After centrifugation, two layers formed. Proteins make up the lower layer, whereas DNA makes up the upper aqueous layer.
- Using pipette, tips, carefully picked the upper aqueous layer containing DNA and poured it into another similarly labeled falcon tube.
- Isopropanol was now added in proportion to the amount of supernatant that cause the DNA to precipitate out and visible as a thread.

- To make a DNA pellet, centrifuge again at 3000rpm and 20°C for 20 minutes.
- The DNA pellet stuck to the falcon tube walls and then the supernatant was completely discarded.
- 5ml of 70% ethanol was added in each tube and were put to centrifuge. Discarded the supernatant again with care, ensuring that DNA remained in the falcon tubes.
- To dry the DNA pellet, the DNA-containing falcon tubes were inverted on the tissue paper.
- After it had dried, TE buffer was poured into each falcon tube according to the amount of DNA thread.
- To dissolve the DNA, each tube was incubated at 37ºC for overnight.

3rd Day of DNA extraction

- At the third day, the DNA was heat-shocked in a water bath at 70°C to prevent it from the denaturation.
- Transferred it in already labeled screw cap tubes.
- This stock of DNA was now stored at 4ºC.

Composition of solutions used for DNA extraction is enlisted in table 2.1.

Table 2.1 Composition of solutions used for DNA extraction

Solutions name	Chemical composition
TE Buffer	10m M TrisHCl, 2 mM EDTA, pH 8.0
TNE buffer	10 mM TrisHCl, 2 mM EDTA, 400 mMNACl
SDS	20%
Proteinase Kinase	10 mg/ml
NACl	6M
Ethanol	70%

2.9 Quantification and Purity Assessment of DNA

Following two methods were used to check the concentration of DNA:

1-Agarose gel electrophoresis estimation with a known standard DNA dilution

The quantity of DNA was determined using UV induced florescence light is in this method. When UV induced fluorescence falls on ethidium bromide intercalated into DNA

bases, we may determine the quantity of DNA by comparing the intensity of fluorescence of DNA samples. The quantity of fluorescence is proportional to the amount of DNA present in a sample. It also allowed the evaluation of nucleic acid integrity at the same time.

2-Nanodrop reader

The quality as well as quantity of DNA was assessed using a Nanodrop reader (Calibri spectrophotometer). A total of 2µl of each DNA sample and blank buffer was used. The purity, quantity and absorbance of the DNA sample were noted. The average DNA concentration was between 5 and 528 ng per microlitre as listed in Table 2.2.

Table 2.2 DNA quantity, absorbance and purity of preeclampsia patients

Sr No.	UAI NO.	DNA (ng/µl)	A 260/280º	A260/230º
1	PE-01	42.39	1.83	2.27
2	PE-02	528.33	1.87	1.45
3	PE-03	84.63	1.75	1.58
4	PE-04	247.27	1.93	2.28
5	PE-05	111.22	1.80	2.32
6	PE-06	145.0	1.88	2.11
7	PE-07	179.01	1.97	1.37
8	PE-08	81.61	1.88	2.14
9	PE-09	176.81	1.87	1.16
10	PE-10	213.38	1.94	1.06
11	PE-11	15.71	2.87	0.09
12	PE-12	127.64	1.98	2.08
13	PE-13	70.21	1.79	2.23
14	PE-14	57.05	2.03	2.49
15	PE-15	334.60	1.95	1.53
16	PE-16	482.35	1.83	1.77
17	PE-17	19.35	1.78	0.29
18	PE-18	215.86	1.72	2.15
19	PE-19	9.66	2.28	0.45

20	PE- 20	97.50	1.81	2.15
21	PE-21	7.54	1.27	0.46
22	PE-22	76.68	2.02	1.52
23	PE-23	67.64	1.87	1.90
24	PE-24	7.94	1.92	0.18
25	PE-25	18.14	1.71	0.34
26	PE-26	227.69	1.86	2.01
27	PE-27	11.39	1.73	1.17
28	PE-28	5.48	0.86	1.07
29	PE-29	12.84	1.36	0.90
30	PE-30	173.97	2.06	2.09
31	PE-31	221.38	1.97	2.01
32	PE-32	20.67	1.68	1.44
33	PE-33	375.80	2.00	2.15
34	PE-34	8.60	1.62	0.30
35	PE-35	89.89	1.97	1.89
36	PE-36	166.19	1.99	2.07
37	PE-37	314.17	2.0	2.17
38	PE-38	13.18	0.66	5.48
39	PE-39	219.18	1.81	1.96
40	PE-40	224.14	1.87	2.22
41	PE-41	144.29	2.00	3.60
42	PE-42	13.59	1.56	0.38
43	PE-43	336.70	1.98	2.41
44	PE-44	87.28	1.92	2.03
45	PE-45	36.97	1.82	2.12
46	PE-46	62.30	1.82	1.09
47	PE-47	43.14	1.80	2.11
48	PE-48	153.49	1.91	1.90
49	PE-49	78.61	2.50	4.3

50	PE-50	29.78	1.73	23.16
51	PE-51	70.33	1.78	1.92
52	PE-52	55.44	1.70	2.74

2.10 Primer Designing

For amplification of the specific target sequence of DNA of the *VDR* gene (exon 2), *FokI* forward primer (CTGGCACTGACTCTGGCTC) and *FokI* reverse primer (ATGGAAACACCTTGCTTCTTCTCCCTC) were used. These primers were adopted from (Lv *et al.*, 2016; Mukhtar *et al.*, 2019). The primer sequence, melting temperature, GC content and product size are mentioned below in Table 2.3.

Table 2.3 *FokI* primers used in amplification of DNA

Primer Name	5'-3' Sequence	Number of Base Pairs	Melting Temperature	GC Content Percentage	Product size
FokI-Forward Primer	CTGGCACTGACTCTGGCTC	19	65-55 TD	63	259bp
FokI-Reverse Primer	ATGGAAACACCTTGCTTCTTCTCCCTC	27		48	

2.11 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was carried out in 200µl PCR tubes that had been labeled previously. Master mix was designed to minimize the error of pipetting which includes the following components; Primers, Taq DNA polymerase, dNTP's, PCR water, PCR buffers KCL and MgCl2. Table 2.4 shows the composition of these components. With one more sample reaction mixture, a master mix for the needed number of samples was prepared. In a Beckman Coulter microfuge, centrifuged the master mix for 1 minute at 3000 rpm. 2.5 µl DNA was added to each PCR tube, followed by master mix based on the number of samples. Tubes were mixed well and spinned for short time.

In Bio-Rad T100 Thermo cycler, PCR reaction was executed with the thermal cycling conditions which are shown in the table 2.5 (Figure 2.1).

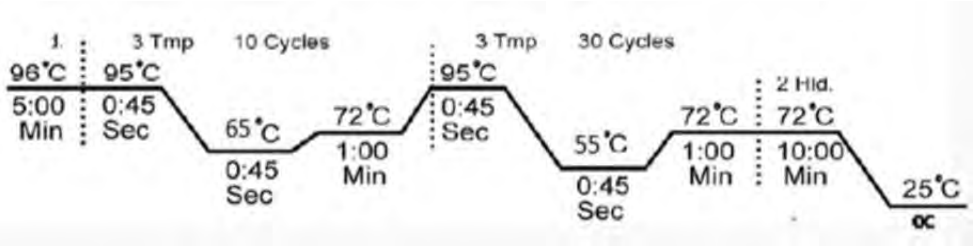

Figure 2.1 Thermocycler conditions for touchdown PCR 65°C→55°C

Table 2.4 Components, composition and quantity of chemical components used in PCR

Sr. No.	Chemical components	Composition	Quantity Used in Single PCR reaction
1	dNTP Mix, 2.5Mm	12.5ul of 100mM each dTTP, dGTP, dCTP, dATP in 450µl PCR water	2.5µl
2	10X Taq Buffer $(NH_4)_2SO_4$	100Mm Tris-HCl (pH 8.8 at 25°C), 200mM $(NH_4)_2SO_4$, 0.8%(v/v) Tween 20	2.5µl
3	25mM $MgCl_2$	-----	2.5µl
4	Forward Primer-10pmol/ µl	4 µl forward primer (100pmol) in 36µl PCR water	0.4µl
5	Reverse Primer-10pmol/ µl	4 µl reverse primer (100pmol) in 36µl PCR water	0.4µl
6	PCR water, Nucleases free	-----	13.7µl
7	DNA	Stored in 0.2mM T.E buffer	2.5µl
8	Taq DNA Polymerase	Enzyme is supplied in: 0.1mM EDTA, 20 mMTris-HCl (pH 8.0), 0.5% (v/v) Nonidet P40, 100mM KCl, 50% (v/v) glycerol and 0.5% (v/v) Tween 20.	0.5µl
Total Reaction Mixture		**25µl**	

Table 2.5 PCR thermo cycling conditions

Sr. No.	Steps	Temperature	Time	Number of cycles
1	Initial Denaturation	96°C	5 min	1
2	Denaturation	95°C	45 sec	34
3	Annealing	65-55°C	45 sec	
4	Extension	72°C	90 sec	
5	Final Extension	72°C	10 min	1
6	Holding	25°C	Infinite	---------

2.11.1 Agarose gel electrophoresis for PCR

When the desired DNA sequence has been amplified by the PCR reaction, DNA was loaded and run on a 2 percent agarose gel to confirm the existence of targeted DNA amplicons. To prepare a 2 percent agarose gel, 1 gram of agarose were added to 50 millilitres of TBE buffer (1X) in a conical flask, which was then covered with aluminium foil and microwaved for 1 minute to mix the gel in the buffer. Then I added 5 microlitres of ethidium bromide into it to see the bands. To avoid the production of bubbles, the hot mixture was carefully poured into the gel caster apparatus before it was polymerized. Composition of gel and buffers used for agarose gel electrophoresis are enlisted in following Table 2.6.

Table 2.6 Composition of buffers as well as gel used for agarose gel electrophoresis

Sr. No.	Solution	Composition	Function
1	**1% Agarose Gel (50 ml)**	10X TBE (5 ml) Distilled water (45 ml) Agarose (0.5 g) Ethidium bromide (5 µl)	To visualize and confirm the presence of DNA bands.
2.	**2% Agarose Gel (50 ml)**	10X TBE (5 ml) Distilled water (45 ml) Agarose (1 g) Ethidium Bromide (5 µl)	To visualize as well as confirm the presence of PCR bands.
3	**Gel Preparing Buffer (10X TBE)**	Tris (54 g) Boric acid (27.5 g) EDTA (3.65 g) Deionized water (500 ml)	1X TBE is prepared from the 10X TBE (stock buffer).
4	**Gel Running Buffer (1X TBE)**	10X TBE (1 part) Distilled water (9 parts)	1X TBE buffer provides the medium for conduction of charges.
5	**Ethidium Bromide (50 ml)**	0.5 g ethidium bromide Autoclaved filtered water (50 ml)	Ethidium bromide is an intercalating agent, when exposed to UV rays it makes DNA visible.
6	**Loading Dye (25 ml)**	Bromo phenol blue (0.0875 g) Sucrose (10 g) Autoclaved filtered water (25 ml)	Loading dye makes the DNA visible in the gel.

To make the wells in which the DNA was loaded, the gel caster contains 16 well combs. It was then left for 20 to 30 minutes to polymerize the gel. Combs were removed as the gel gets polymerized and placed it in a gel tank with (1X) TBE buffer. 2 microlitres PCR product mixed with 2 microlitres loading dye i.e. Bromophenol blue were loaded into the wells carefully with the help of micropipette. A ladder (100bp DNA ladder, Thermo Scientific) was also loaded into the first well as a size standard for detecting bands of various sizes.

In a horizontal agarose gel electrophoresis apparatus (CS-3000V, Cleaver Scientific Limited) containing 1X TBE buffer, electrophoresis was performed for 25 minutes at 120'V' and 120'A'. In a gel documentation system, the bands were visualized by placing a gel over UV transilluminator (Cleaver) as well as image was captured by a Digital Camera.

2.12 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) was used to detect the T/C polymorphism in the start codon (ATG) of exon 2, which is the translation initiation site of

VDR gene. For this, we chose the enzyme restriction endonuclease *FokI* from the restriction mapper based on the sequence of the mutated region in exon 2. The enzyme was ordered from ThermoFisher Scientific Company. It makes a staggered cut on the double stranded DNA at 9 and 13 nucleotides away from 5‘-GGATG-3‘ sequence in the targeted region as shown in figure 2.2. As stated in table 2.3, primers were designed to amplify the required sequence of exon 2 of *VDR* gene. Following is the recognition site of *FokI* restriction enzyme (Figure 2.2):

Figure 2.2 *Fok*I restriction site. *Fok*I cut 9 bp away from GGATG sequence on the 5′ strand and 13 bp away from CCTAC sequence on 3′ strand, as shown by the arrows

Following method was used for digestion with *FokI* Fast Digest restriction enzyme:

2.12.1 Methodology of enzyme digestion

a) Preparation of reaction mixture

- PCR tubes were labeled.
- 5 µl of PCR product were added in each tube.
- Then added the 1µl Fast digest buffer, 0.5µl Fast digest *FokI* enzyme and 8.5µl PCR water in each sample product.
- Centrifuged it at 3000 rpm for 1 minute in a Beckman Coulter microcentrifuge.
- Tubes were then placed in incubator for 30 minutes at 37ºC.
- Then 0.15 µl of 20% SDS (sodium dodecyl sulphate) were added into each tube in order to degrade the enzyme.
- Again centrifuged the tubes at 3000 rpm for 1 minute.
- Tubes were then heated at 70ºC for 10 minutes in a PCR machine.

b) 2.5% Agarose gel electrophoresis

After digestion, the same method as stated in before (2.5.5) was used to check digestion products on a 2.5 percent agarose gel. A 100-base-pair DNA ladder was also loaded to assess the size of the bands after the digestion. To clearly distinguish the two cut bands, electrophoresis was firstly performed at 100 voltage for 5 minutes then at 70 voltage for 1 hour.

c) Expected outcomes of RFLP

Variant c.2T>C of single nucleotide i.e. T is a form of substitution. Fast Digest *FokI* is the enzyme we chose, was supposed to cleave wild type sequence. The 259 bp product is amplified by the designed primer and there are only one cut sites in this amplified sequence. The wild type sequence will be cleaved at the mutation site, resulting in two different bands, each 197 and 62 bp long. The sequence with the c.2T>C variant, on the other hand, will not be cut into two bands of 197 and 62 bp. In case of one c.2T>C variant allele and one wild type allele, resulted in three different bands of 259, 197 and 62 bp long.

RESULTS

Section A-Results of Epidemiological Studies

3.1 Study Design - Case Control Study

The present study was performed at gynae ward of CMH hospital during a period of April to July 2021. For this study, diagnosed patients with PE having systolic and diastolic blood pressure of ≥140/90 respectively along with proteinuria ≥300 mg per day or ≥1+ on dipstick calculated on two different occasions at least 6 hours apart and age ≥17 years were included in current investigation while the non-preeclamptic women having blood pressure ≤125/85 as well as no proteinuria were taken as a control in this study. Individuals having abnormal embryo seen in ultrasound, severe kidney disorder, malignant syndrome and consumption of immunosuppression and anticancer treatment were excluded. Total of 60 cases and 711 control pregnant women were recruited for this study. Out of all pregnant women 8.4% were affected with PE in 4 months.

Each participant in the case and control signed a consent form and these forms were kept as a record. Participants were interviewed using a designed questionnaire to obtain information on various parameters and risk factors. As the study design was case control so according to criteria 60 cases and 240 age matched controls were selected for further analysis of risk factors and maternal as well as fetal outcomes.

3.2 Baseline Characteristics of Cases and Controls-Frequency and Percentages

Various demographic data of participants were recorded in addition to the medical and lifestyle factors in order to determine the risk of various factors. The mean age of the study population (cases and controls) was 29.23±5.944 years. Cases were more likely to be primiparous and have family history of hypertension, with more previous fetal complications, lower intake of supplements, greater use of infertility treatment, with more previous miscarriages, less consanguinity, higher smokers, less educated, more stillbirth, greater neonatal morbidity, lower number of deceased child, higher incidence of gestational diabetes, history of chronic hypertension, pregestational diabetes and pregnancy induced hypertension (PIH) as described in table 3.1.

Most of the cases were with blood group O positive (45% cases, 25.8% controls), have greater body mass index (BMI), have high blood pressure, have low infant weight, elevated alkaline phosphatase (ALP), alanine transaminase (ALT), serum creatinine,

bilirubin and urea, less hemoglobin and presentation of high proteinuria as compared to control subjects as listed in Table 3.1.

Table 3.1 shows the standard characteristics of the individuals of both cases and controls included in this investigation.

Table 3.1 Baseline characteristics of PE patients and controls

Baseline Characteristics		Cases		Controls	
		Frequency	**Percentage**	**Frequency**	**Percentage**
Age	**≤ 21**	6	10.0	24	10.0
	22-29	26	43.3	104	43.3
	30-37	23	38.3	92	38.3
	≥ 38	5	8.3	20	8.3
Age at Marriage	**≤ 20**	28	46.7	101	42.1
	21-28	28	46.7	123	51.3
	≥ 29	4	6.7	16	6.7
Gestational Age (weeks)	**≤ 30**	10	16.7	4	1.7
	31-38	48	80.0	193	80.4
	≥ 39	2	3.3	43	17.9
Education	**Uneducated**	17	28.3	59	24.6
	Primary	9	15.0	51	21.3
	Secondary	17	28.3	70	29.2
	Higher Education	17	28.3	60	25.0
Occupation	**Student**	1	1.7	7	2.9
	Housewife	46	76.7	207	86.3
	Working	13	21.7	26	10.8
Cousin Marriage	**Yes**	34	56.7	138	57.5
	No	26	43.3	102	42.5
Parity	**Primiparity**	21	35.0	58	24.2
	Multiparity	20	33.3	107	44.6
	Grandparity	19	31.7	75	31.3
Miscarriage	**No**	37	61.7	164	68.3
	1-2	16	26.7	69	28.8
	Many(3-5)	7	11.7	7	2.9
Infertility Treatment	**Yes**	8	13.3	19	7.9
	No	52	86.7	221	92.1
Patient Smoking	**Yes**	2	3.3	2	.8
	No	58	96.7	238	99.2
Blood Group	**N/A**	12	20.0	21	8.8
	A positive	10	16.7	60	25.0
	A negative	1	1.7	4	1.7
	B positive	6	10.0	65	27.1
	B negative	0	0	6	2.5
	O positive	27	45.0	62	25.8
	O negative	2	3.3	6	2.5
	AB positive	2	3.3	16	6.7
Supplements Intake	**Yes**	45	75.0	205	85.4
	No	15	25.0	35	14.6
Singlets/Twins	**Single**	58	96.7	237	98.8
	Twins	2	3.3	3	1.3
Previous Fetal Complications	**Yes**	3	5.0	6	2.5
	No	57	95.0	234	97.5

Family History	**Yes**	45	75.0	43	17.9
	No	15	25.0	197	82.1
Current Delivery Mode	**Cesarean**	51	85.0	182	75.8
	Vaginal	9	15.0	58	24.2
Previous Mode of Delivery	**No**	21	35.0	70	29.2
	Cesarean	17	28.3	107	44.6
	Vaginal	22	36.7	63	26.3
BMI	**< 18.5**	2	3.3	22	9.2
	18.5-24.9	15	25.0	109	45.4
	25-29.9	21	35.0	64	26.7
	> 30	22	36.7	45	18.8
Stillbirth	**Yes**	8	13.3	5	2.1
	No	52	86.7	235	97.9
Infant Sex	**N/A**	1	1.7	1	.4
	Boy	27	45.0	119	49.6
	Girl	31	51.7	118	49.2
	Both Girls	0	0	2	0.8
	Both Boys	1	1.7	0	0
Infant Weight (kg)	**N/A**	20	33.3	9	3.8
	≤ 2	3	5.0	6	2.5
	2.1-3.5	36	60.0	202	84.2
	≥ 3.6	1	1.7	23	9.6
Neonatal Morbidity	**Yes**	5	8.3	8	3.3
	No	55	91.7	232	96.7
Geographic Distribution of Participants	**Muzaffarabad**	47	78.3	203	84.6
	Neelum Valley	3	5.0	10	4.2
	Bagh	2	3.3	11	4.6
	Hattian Bala	7	11.7	11	4.6
	Other	1	1.7	5	2.1
Deceased Child History	**Yes**	1	1.7	21	8.8
	No	59	98.3	219	91.3
GDM	**Yes**	10	16.7	20	8.3
	No	50	83.3	220	91.7
Chronic Hypertension	**Yes**	6	10.0	8	3.3
	No	54	90.0	232	96.7
Pregestational Diabetes	**Yes**	1	1.7	2	0.8
	No	59	98.3	238	99.2
Previous History of PIH	**Yes**	8	13.3	21	8.8
	No	52	86.7	219	91.3
Systolic Blood Pressure	**Low**	1	1.7	29	12.1
	Normal	1	1.7	160	66.7
	High	58	96.7	51	21.3
Diastolic Blood Pressure	**Low**	0	0	42	17.5
	Normal	2	3.3	164	68.3
	High	58	96.7	34	14.2
Platelets	**Low**	7	11.7	2	0.8
	Normal	50	83.3	236	98.3
	High	3	5.0	2	0.8
Alkaline Phosphatase (ALP)	**Normal**	18	30.0	234	97.5
	High	42	70.0	6	2.5
Alanine Transaminase (ALT)	**Normal**	35	58.3	239	99.6
	High	25	41.7	1	0.4

Serum Creatinine	**Low**	8	13.3	50	20.8
	Normal	37	61.7	190	79.2
	High	15	25.0	0	0
Serum Urea	**Low**	3	5.0	18	7.5
	Normal	44	73.3	212	88.3
	High	13	21.7	10	4.2
Urinary Protein	**300(+)**	8	13.3	240	100.0
	600(++)	30	50.0	0	0
	900(+++)	19	31.7	0	0
	> 900	3	5.0	0	0
Blood Hemoglobin	**Low**	41	68.3	81	33.8
	Normal	19	31.7	159	66.3
Serum Bilirubin	**Normal**	55	91.7	240	100.0
	High	5	8.3	0	0

Mean values of a continuous variable including age, gestational age, age at marriage, BMI and infant age is shown in table 3.2 for both cases and controls. Average value of age was 29.23±5.944 years and 29.23±5.906 years in cases and controls respectively. Mean value of gestational age was respectively documented as 34.70±3.258 and 37.22±2.115 weeks in cases and controls, so cases were observed to have less gestational age. For cases and controls, average value of age at marriage was respectively observed as 21.93±3.973 and 21.99±3.893 years. Average value of continuous variable BMI was 28.32±5.580 kg/m^2 for cases and 25.03±5.844 kg/m^2 for controls which shows that PE patients were obese. Mean value of infant weight was respectively found as 1.84±1.379 and 2.91±1.460 kg for cases and controls, which depicts that infant weight of PE patients were low in comparison to controls. In section 3.5, the details of each baseline characteristics are graphically presented in Figures 3.11-3.37.

Table 3.2 Mean Value of continuous variable of PE patients and Controls

Characteristics	Cases	Controls
Age± SD (years)	29.23±5.944	29.23±5.906
Gestational Age± SD (weeks)	34.70±3.258	37.22±2.115
Age At Marriage± SD (years)	21.93±3.973	21.99±3.893
BMI± SD (kg/m^2)	28.32±5.580	25.03±5.844
Infant Weight± SD (kg)	1.84±1.379	2.91±1.460

3.3 Clinical and pathological data of PE patients and controls

Table 3.3 presents the comparative statistical analysis of systolic blood pressure, diastolic blood pressure, blood hemoglobin, platelets count, alkaline phosphatase, alanine transaminase, serum bilirubin, serum creatinine, serum urea, proteinuria among PE patients

and controls. The mean Systolic Blood Pressure was 145.33±15.78 mmHg and 121.21±7.64 mmHg in cases and controls respectively (p<0.0001). The mean diastolic blood pressure was 95.67±7.45 mmHg and 79.71±6.56 mmHg in cases and controls respectively (p<0.0001). Average systolic and diastolic blood pressure in cases was found higher than the controls.

Table 3.3 Clinical and pathological data of PE patients and controls

Clinical and Pathological Data	Cases N=60	Controls N=240	t-value	p-value	95% CI of the difference	
					Lower	Upper
Systolic Blood Pressure (mmHg)	145.33±15.78	121.21±7.64	17.049	<0.0001	21.34	26.91
Diastolic Blood Pressure (mmHg)	95.67±7.45	79.71±6.56	16.395	<0.0001	14.04	17.87
Blood Hemoglobin (g/dl)	11.16±1.55	12.10±1.10	-5.425	<0.0001	-1.28	-0.60
Platelets Count (10^3/μL)	265.50±86.52	286.87±62.30	-2.184	0.030	-40.62	-2.11
Alkaline Phosphatase (IU/L)	305.50±132.64	138.52±81.36	12.338	<0.0001	140.35	193.62
Alanine Transaminase (IU/L)	38.88±29.41	23.82±16.54	5.282	<0.0001	9.45	20.68
Serum Bilirubin (μmol/L)	12.03±3.55	9.56±3.61	4.76	<0.0001	1.45	3.49
Serum Creatinine (mmol/L)	99.95±26.54	89.38±15.77	3.977	<0.0001	5.34	15.80
Serum Urea (mmol/L)	5.58±1.50	4.83±1.39	3.682	<0.0001	0.35	1.154
Urinary Protein (mg/24hrs)	734.83±458.19	300.00±0.00	14.777	<0.0001	376.92	492.74

N: number of participants

Mean blood hemoglobin in cases was 11.16±1.55 g/dl and in controls it was 12.10±1.10 g/dl (p<0.0001) as enlisted in table 3.3. Mean value of blood Hb in cases was found smaller than the controls. Mean platelets count in cases was 265.50±86.52 (10^3/μL) while in controls it was 286.87±62.30 (10^3/μL) (p=0.03). Average value of platelets count in cases was found relatively smaller than the controls. Average value of alkaline phosphatase was observed as 305.50±132.64 (IU/L) in cases and 138.52±81.36 (IU/L) in controls (p<0.0001). Mean value of alanaine transaminase was observed as 38.88±29.41 (IU/L) and 23.82±16.54 (IU/L) in cases and controls respectively (p<0.0001). Mean value of alkaline phosphatase and alanine transaminase in cases were elevated in comparison to controls. Mean serum bilirubin was 12.03±3.55 μmol/L in cases and 9.56±3.61 μmol/L in controls (p<0.0001). Mean serum creatinine was 99.95±26.54 mmol/L and 89.38±15.77 mmol/L in cases and controls respectively (p<0.0001). Average value of serum urea was 5.58±1.50 mmol/L in cases while 4.83±1.39 mmol/L in controls (p<0.0001). Mean value

of serum bilirubin, creatinine and urea were relatively found higher than controls. Mean value of urinary proteins was observed as 734.83±458.19 mg/24hrs and 300.00±0.00 mg/24hrs in cases and controls respectively ($p<0.0001$). Average value of urinary proteins were found elevated in PE patients than controls.

3.4 Graphical Representation of Clinical Characters in PE Patients and Controls

3.4.1 Systolic blood pressure

Cases and controls with systolic blood pressure were categorized into three group; low, high and normal. Percentage of controls with systolic blood pressure were found to follow the order as normal (66.7%) > high (21.3%) > low (12.1%). Higher systolic blood pressure were commonly found in cases than controls as high group have greatest percentage of cases (96.7%), while both low and normal group have 1.7% cases (Figure 3.1).

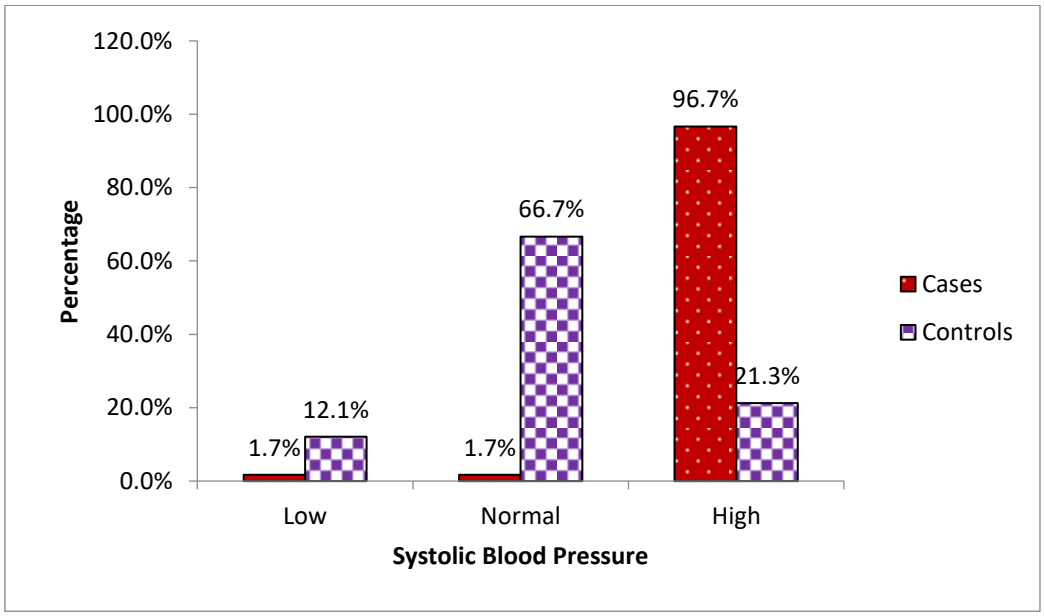

Figure 3.1 Percentage of cases and controls categorized on the basis of systolic blood pressure

3.4.2 Diastolic blood pressure

Cases and controls with diastolic blood pressure were categorized into three group; low, high and normal. Percentage of controls with diastolic blood pressure were found to follow the order as normal (68.3%) > low (17.5%) > high (14.2%). High diastolic blood pressure were commonly found in cases than controls as high group have greatest percentage of cases (96.7%) , while both low and normal groups respectively have 0 and 3.3% cases (Figure 3.2).

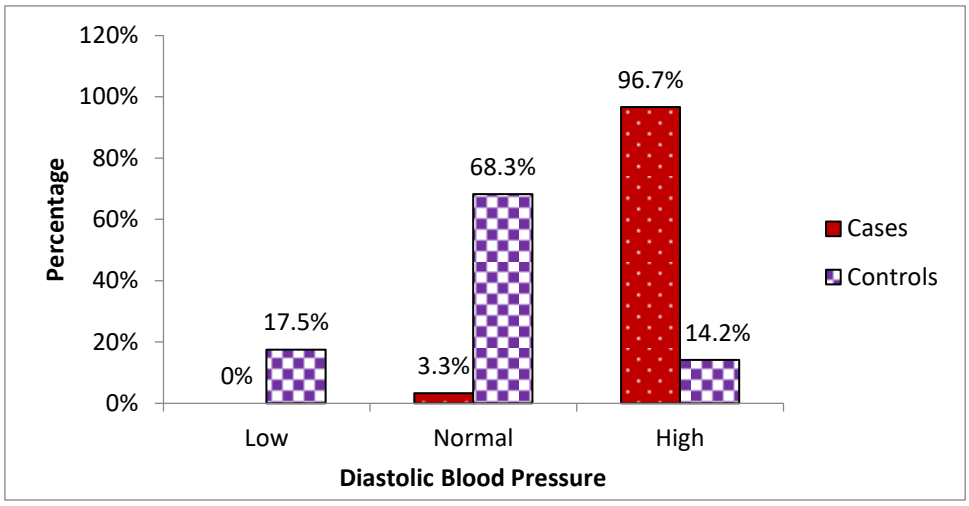

Figure 3.2 Percentage of cases and controls categorized on the basis of diastolic blood pressure

3.4.3 Platelets count

Cases and controls with platelets counts were categorized into three group; low, high and normal. Normal group have highest percentage of platelets counts as compared to other groups i.e. low and high, both for cases and control. Cases with platelets count were follow the pattern as normal (83.3%) > low (11.7%) > high (5%). On the other hand, controls were found with greatest percentage of platelets count (98.3%) for normal group; while both high and low group have 0.8% platelets count (Figure 3.3).

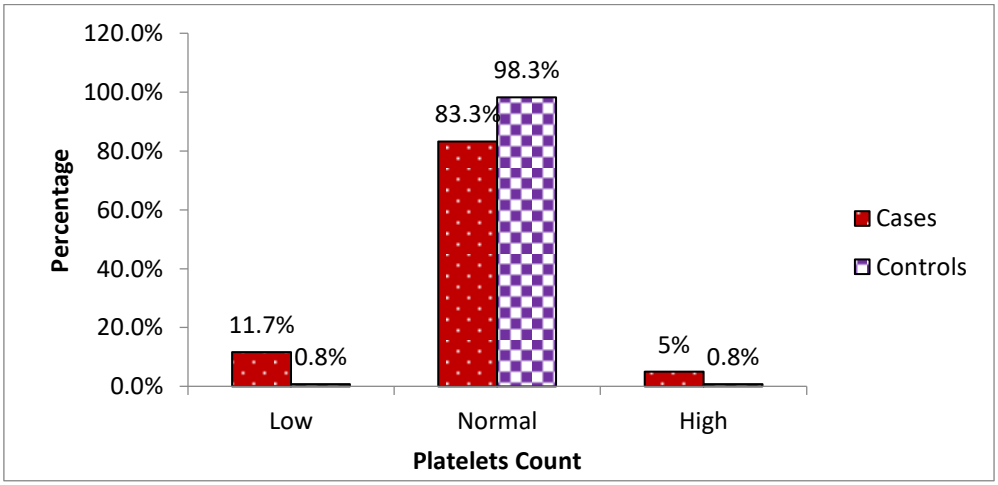

Figure 3.3 Percentage of cases and controls categorized on the basis of platelets count

3.4.4 Alkaline phosphatase

Cases and control were categorized into normal and high group. Cases were observed with greatest percentage of alkaline phosphatase (70%) that fall in high group and 30% being smallest which lie under normal group. On the other hand, control with alkaline phosphatase were found to occupied the groups as normal (97.5%) > high (2.5%).

Greater percentage of control (97.5%) was lie under normal group as compared to cases (30%) (Figure 3.4).

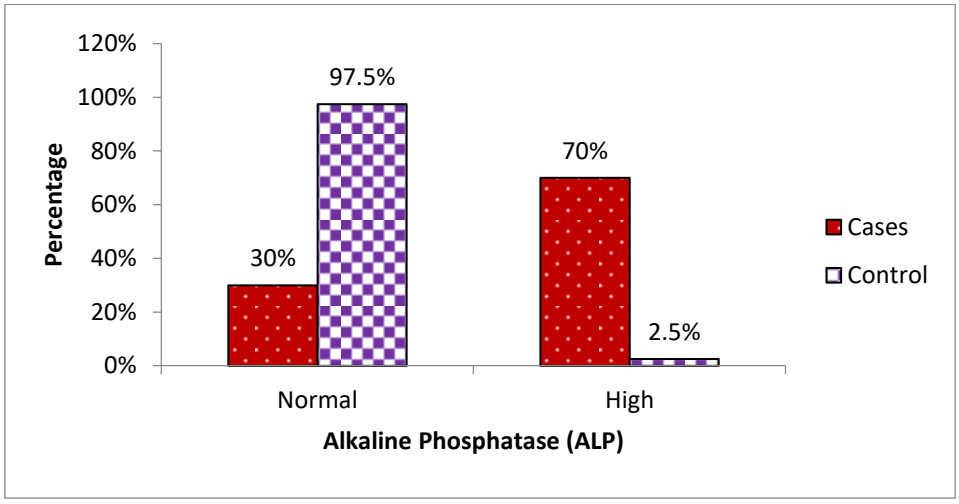

Figure 3.4 Percentage of cases and controls categorized on the basis of alkaline phosphatase

3.4.5 Alanine transaminase

Cases and control were divided into normal and high group. About 58.3% cases with alanine transaminase fall under normal group, while 41.7% fall within high group. Control with alanine transaminase was observed to have 99.6% that fall under normal group and 0.4% of high group (Figure 3.5).

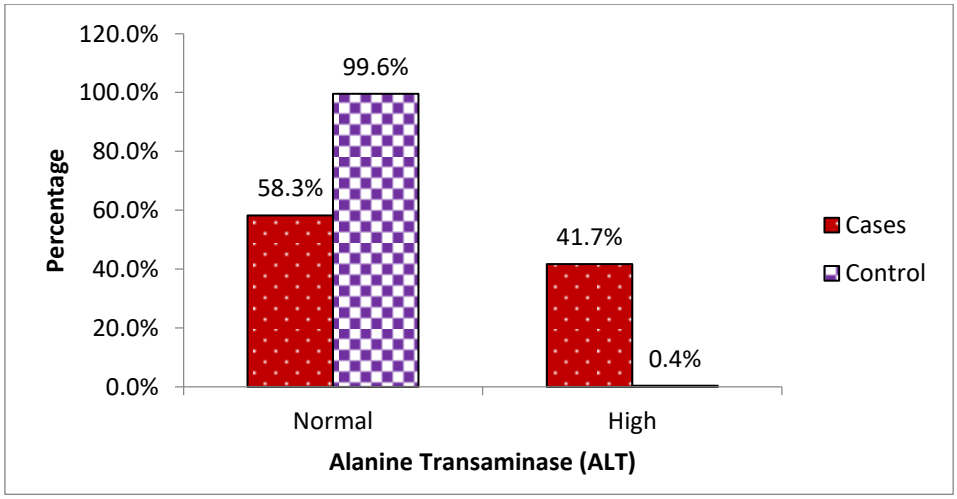

Figure 3.5 Percentage of cases and controls categorized on the basis of alanine transaminase

3.4.6 Blood hemoglobin

Cases and control were placed into low and normal group. Low group have 68.3% cases and 33.8% control. Normal group consist of 31.7% cases and 66.3% control. Majority of cases were observed with less blood hemoglobin (Figure 3.6).

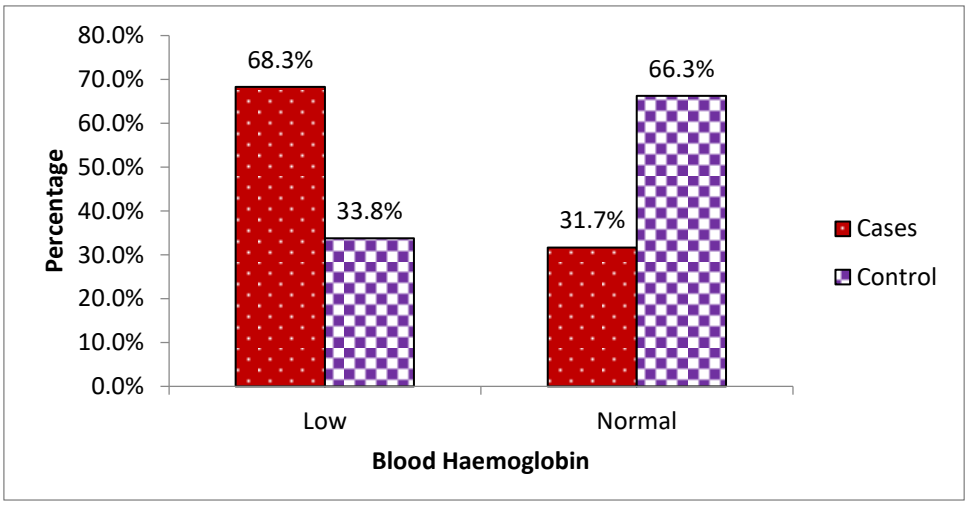

Figure 3.6 Percentage of cases and controls categorized on the basis of blood hemoglobin

3.4.7 Serum urea

Cases and control were subdivided into three groups; low, normal and high. Cases percentage with serum urea was follow the order as normal (73.3%) >high (21.7%) > low (5%). Control percentage with serum urea were found to follow the pattern as normal (88.3%) > low (7.5%) >high (4.2%). The greatest percentage of cases (73.3%) was found to have normal serum urea (Figure 3.7).

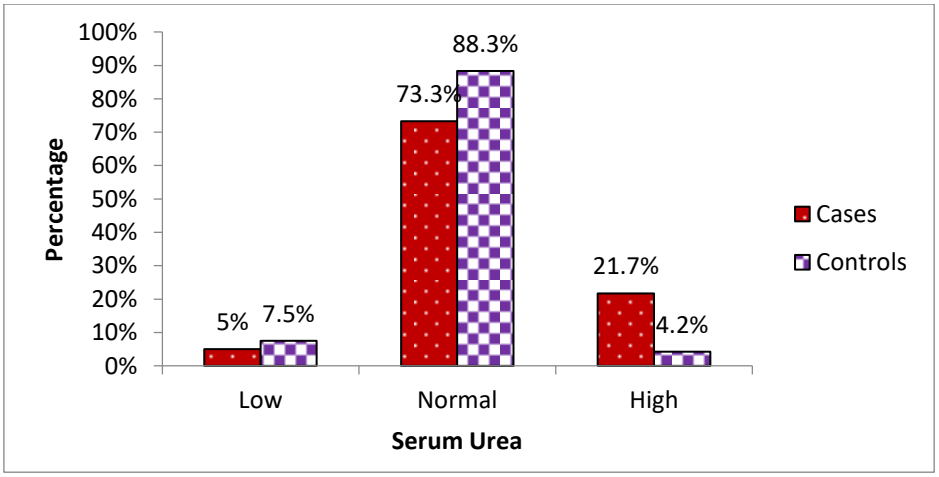

Figure 3.7 Percentage of cases and controls categorized on the basis of serum urea

3.4.8 Serum creatinine

Cases and control were subdivided into three groups; low, normal and high. Cases percentage with serum creatinine was follow the order as normal (61.7%) > high (25%) > low (13.3%). Control percentage with serum creatinine were found to follow the pattern as normal (79.2%) > low (20.8%) > high (0%). Most cases were found to have normal serum creatinine (Figure 3.8).

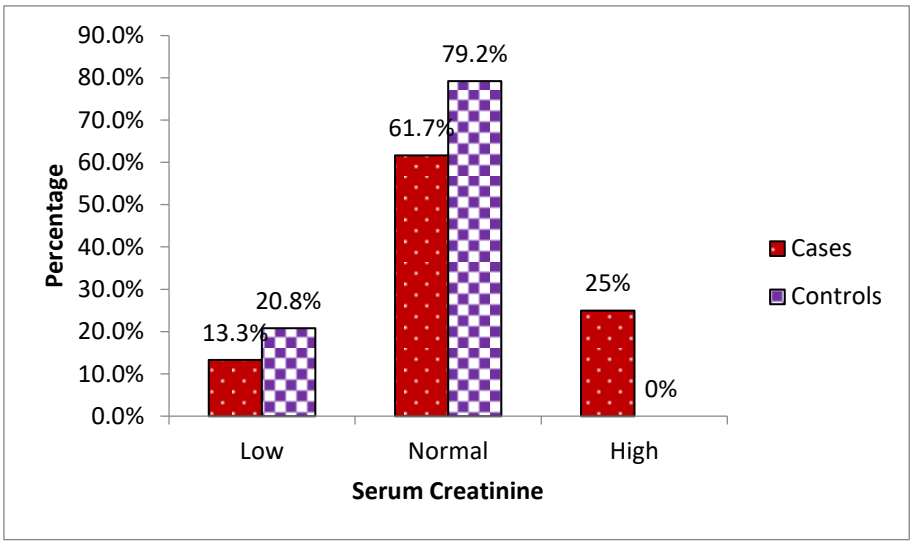

Figure 3.8 Percentage of cases and controls categorized on the basis of serum creatinine

3.4.9 Serum bilirubin

Cases and control with serum bilirubin were divided into normal and high group. Normal group have 100% control and 91.7% cases. On the other hand, high group have 8.3% cases and 0% control. So, greater percentage of cases was observed to have normal serum bilirubin (Figure 3.9).

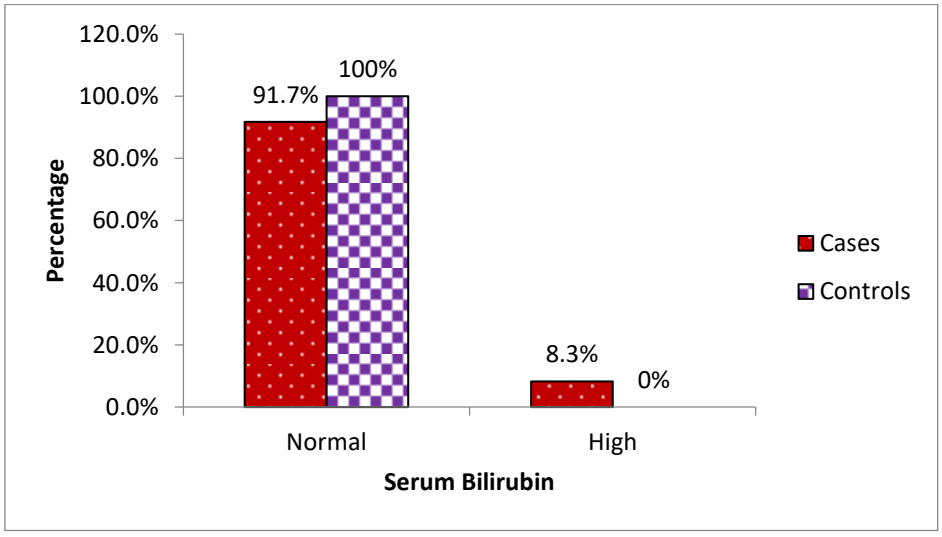

Figure 3.9 Percentage of cases and controls categorized on the basis of serum bilirubin

3.4.10 Urinary protein

Cases and control were divided into four subgroups; (300 mg/day), (600 mg/day), (900 mg/day) and (> 900 mg/day). Subgroup (300) for urinary protein has 100% control, while rest of subgroup was reported with zero percentage. Subgroups (300), (600), (900) and (> 900) with urinary protein has 13.3%, 50%, 31.7% and 5% cases respectively.

Greatest percentage of cases with urinary protein was observed for subgroup (600) and smallest for (> 900) subgroup (Figure 3.10).

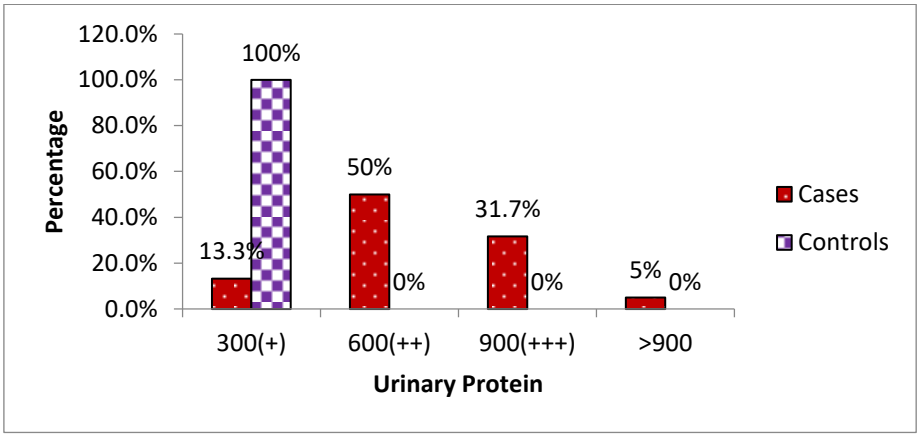

Figure 3.10 Percentage of cases and controls categorized on the basis of urinary protein

3.5 Graphical Representation of Demographic Characters

3.5.1 Age

In demographic features, age was divided into four subgroups viz. ≤ 21, 22-29, 30-37, and ≥38. The majority of patients diagnosed with PE are between the ages of 22 and 29. Less than 9% of total PE cases belong to age group ≥38 (Figure 3.11).

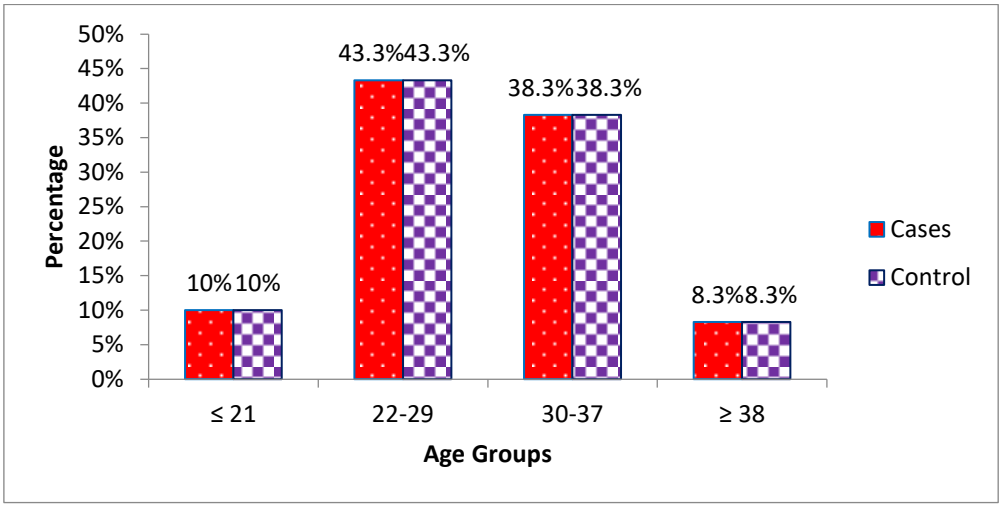

Figure 3.11 A bar chart depicting the percentage of different age groups of research participants

3.5.2 Profession

Participants were divided into 3 sub-groups based on their occupation: student, housewives, working women and found to follow the following order: housewives > working women > students. Majority of the study's participants were housewives (See figure 3.12).

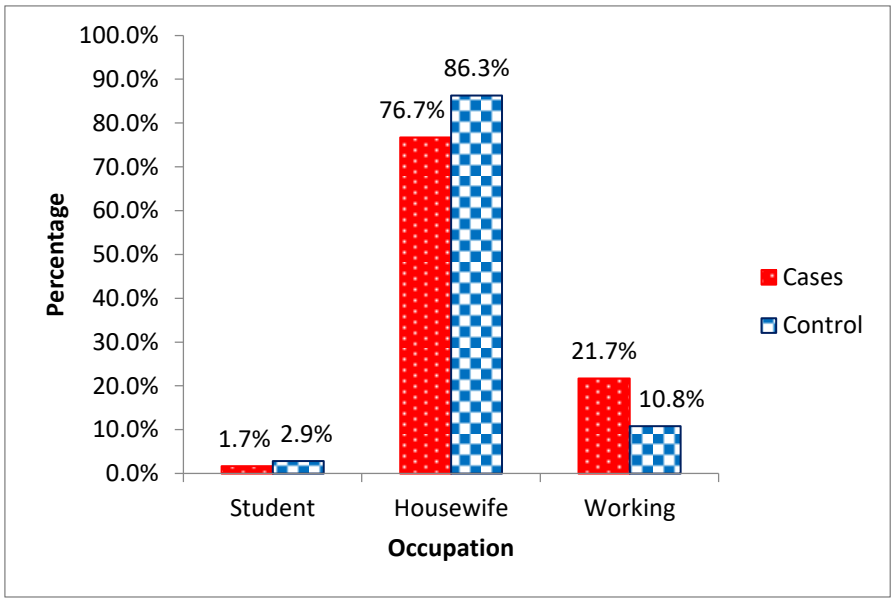

Figure 3.12 Representation of women's professional status in a PE study

3.5.3 Educational Status

In this study, patients were educated as at primary level, secondary level and with higher graduate level. About 28% PE patients were uneducated while others were educated which is quite high in comparison to control (Figure 3.13).

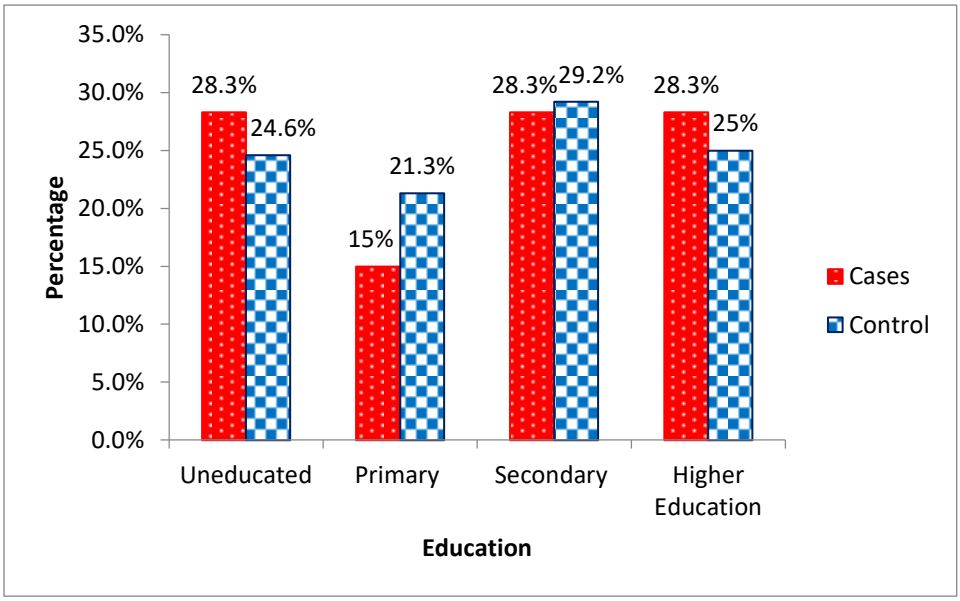

Figure 3.13 Educational status of the patients and controls in current investigation

3.5.4 Family History

In comparison to controls, 75% of cases had a positive family history of disease, which is significantly high compared to controls i.e. 17.9% (Figure 3.14).

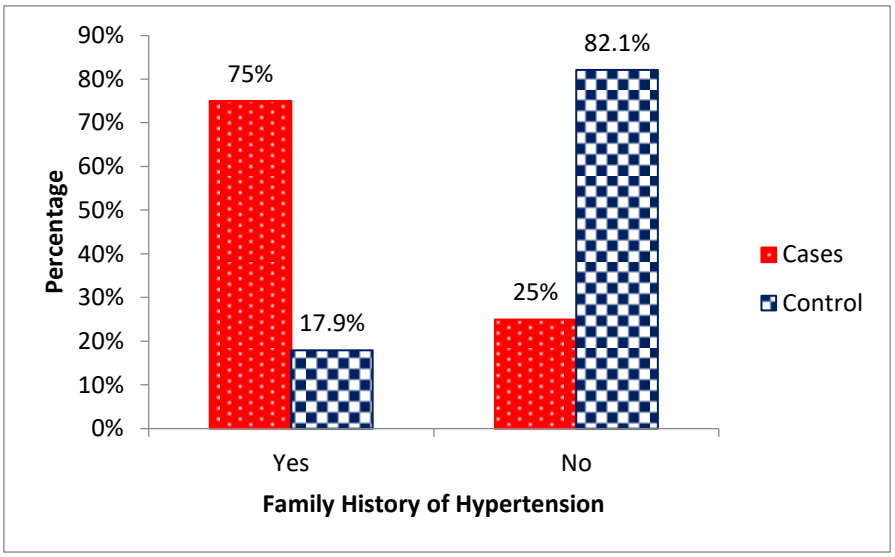

Figure 3.14 Proportional representation of patients and controls according to the family history of hypertension

3.5.5 Parity

To determine the impact of primiparity on the development of PE, women were divided into groups on the basis of number of live births. There were 35% primiparous PE patients while only 24% were primiparous controls in this study. A higher percentage of the participants were pregnant. The majority of female participants were multiparous, while there were a small proportion of participants that was grandparous. The data obtained is shown in figure 3.15.

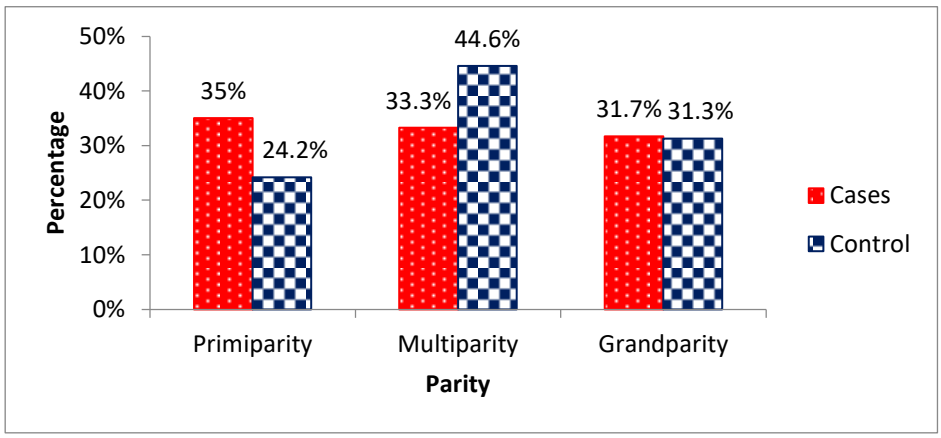

Figure 3.15 Representation of parity percentage between the cases and controls

3.5.6 Body Mass Index

PE patients were divided into four groups according to their body mass index (BMI), which was calculated using the current weight and height of participant's: underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2), and obese (>30 kg/m^2). Out of 60 PE patients, 3.3% were underweight, 25% were normal

weight, 35% were overweight, and 36.7% were obese. 9.2% of the controls were underweight, 45.4% were normal weight, 26.7% were overweight, and 18.8% were obese. Majority of the cases were obese. The data obtained is shown in figure 3.16.

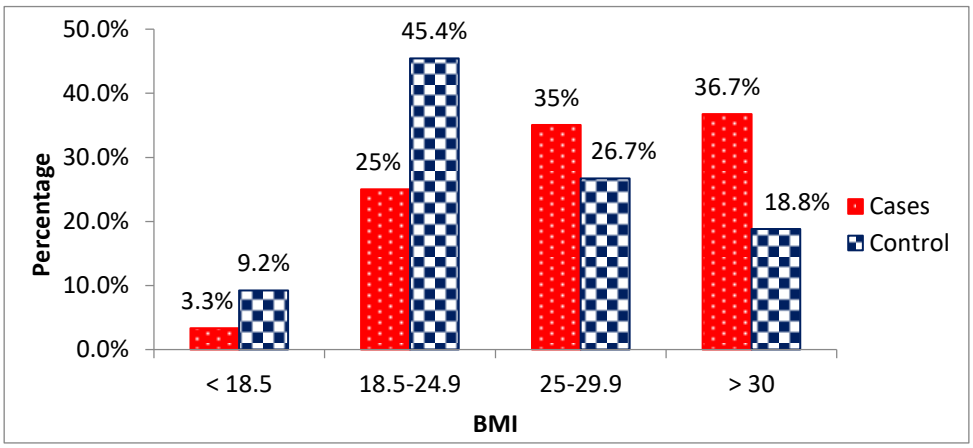

Figure 3.16 A graph depicting the percentages of PE patients and controls in terms of their BMI categories

3.5.7 Smoking Habit

Patients were also checked for their smoking habits and it was found that about 86.7% of the 60 PE patients were nonsmokers, while 13.3% were smokers and 92.1% of the controls were nonsmokers, while 7.9% were smokers (Figure 3.17).

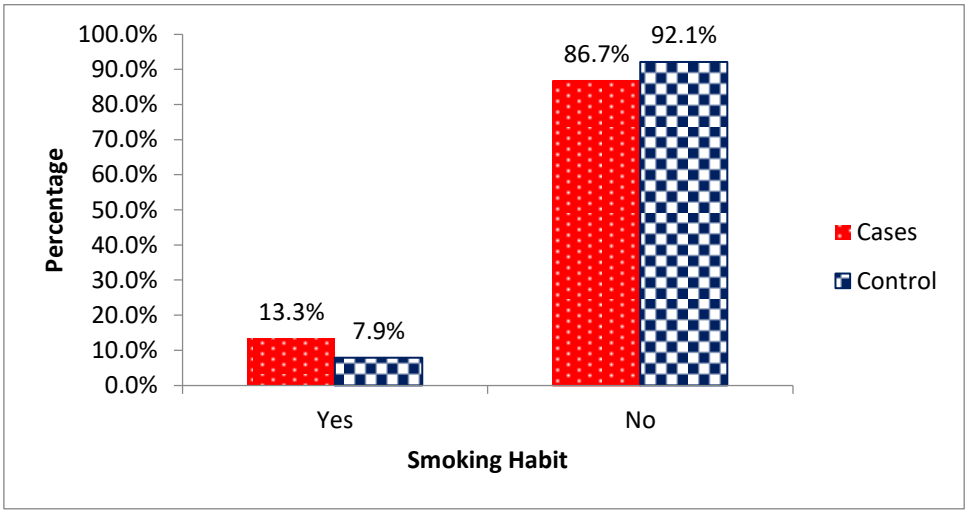

Figure 3.17 Percentage of smoking habit among PE patients and controls

3.5.8 Number of miscarriages

In comparison to cases, there were more women in control group who had never suffered a miscarriage in their lives. In compared to controls, there were more women in cases who had miscarriages during their lifetime. The number of miscarriages among the cases and controls is depicted in figure 3.18.

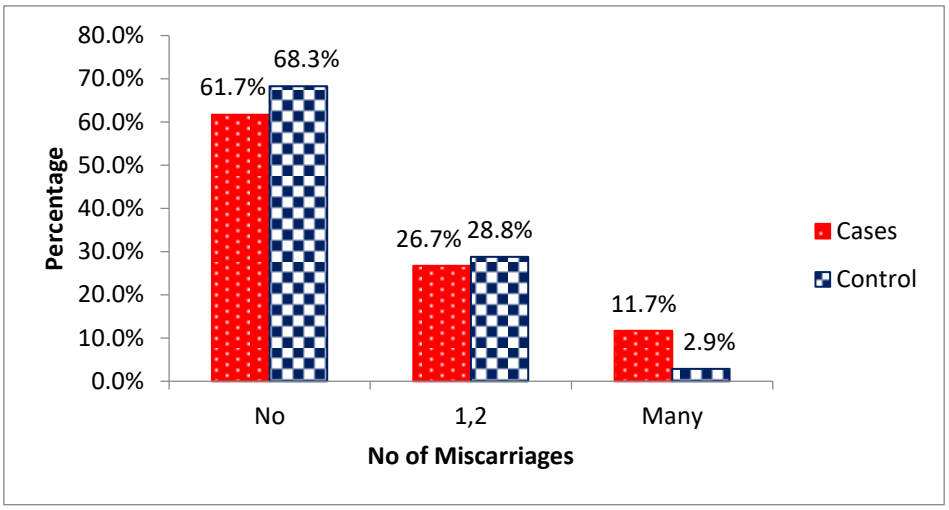

Figure 3.18 A graph depicting the number of miscarriages in the cases and controls

3.5.9 Hypertension

Studies examining the link between chronic hypertension and the risk of PE have produced conflicted results. As indicated in figure 3.19, chronic hypertension was found more common in cases than in controls.

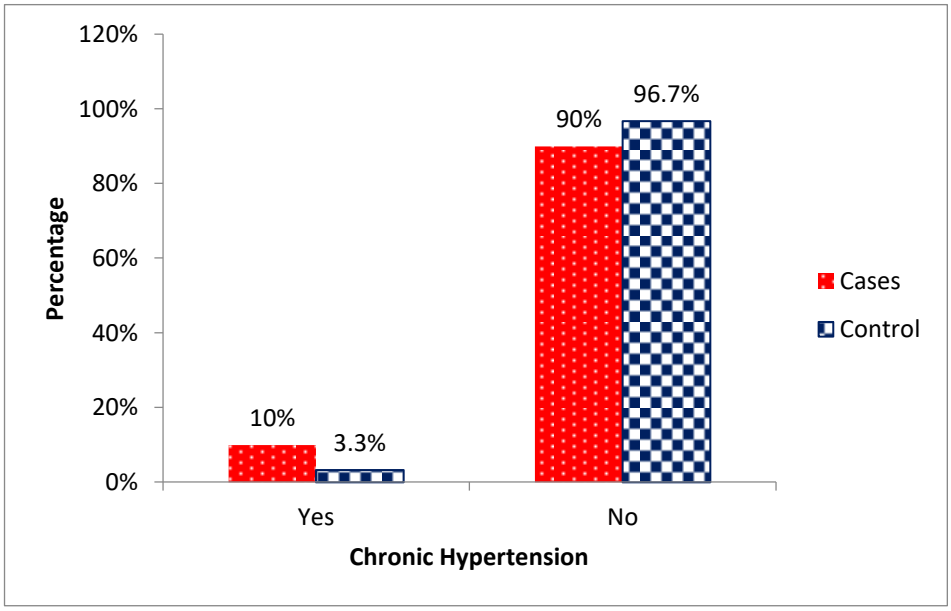

Figure 3.19 Percentage of cases and controls categorized on the basis of chronic hypertension

3.5.10 Age at Marriage

Patients were investigated for relationship between marriage age (years) and PE or controls, which are categorized as ≤ 20 years, 21-28 years and ≥29 years. About 46.7% cases were found for both age groups ≤ 20 years, and 21-28 years, in comparison to controls that were found as 42.1% and 51.3% respectively. Whereas, age group ≥29 years was observed with similar percentage 6.7% for both cases and controls (Figure 3.20).

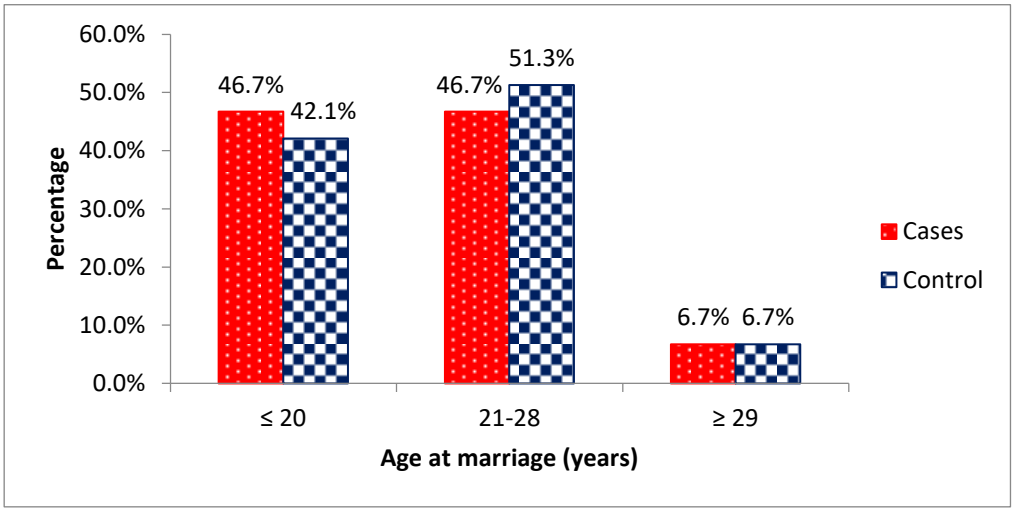

Figure 3.20 Percentage of cases and controls categorized on the basis of their married age

3.5.11 Gestational Age

Gestational age is subdivided as ≤30 weeks, 31-38 weeks and ≥39 weeks that were checked for cases and controls. For gestational age ≤30 weeks; cases was reported as 16.7%, while control were 1.7%. For gestational age 31-38 weeks; both cases and controls were observed with somewhat similar percentage 80.0% and 80.04% respectively. But 17.9% controls were observed in comparison to case i.e. 3.3% for gestational age ≥39 weeks. For gestational age of ≤30 weeks; percentage of cases were surprisingly found higher than controls (Figure 3.21).

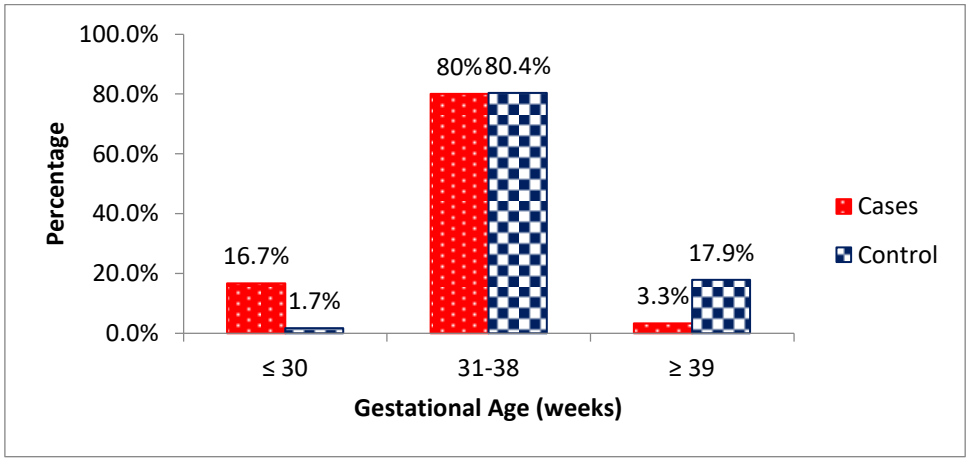

Figure 3.21 Percentage of cases and controls categorized on the basis of gestational age

3.5.12 Singlet/Twins

Study examined the percentage of cases and controls patients for Singlets/Twin babies. It was observed that 96.7% singlets were found for cases and 98.8% for control. But, 3.3% and 1.3% twins were reported respectively for the cases and control (Figure 3.22).

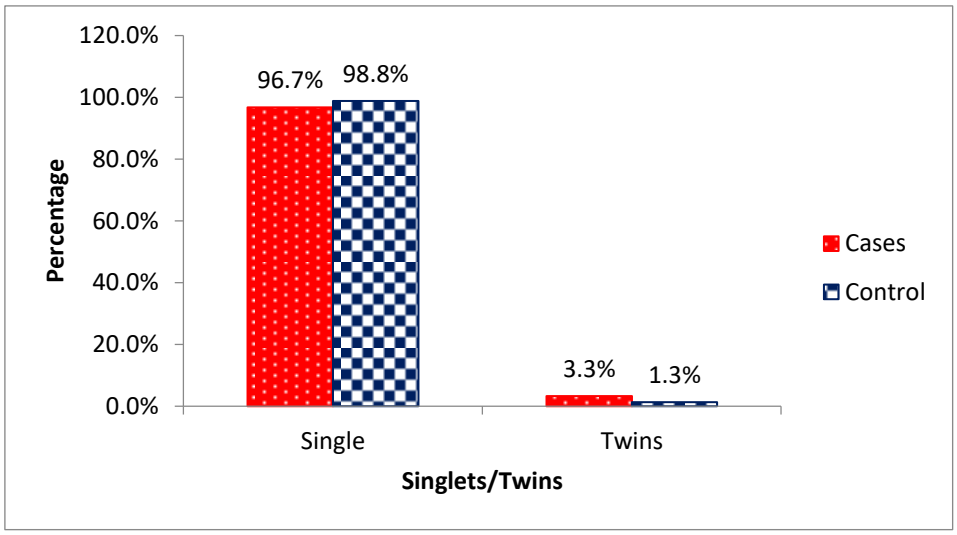

Figure 3.22 Percentage of cases and controls categorized on the basis of singlets/twins

3.5.13 Cousin Marriage

In current study, it is examined that patients with 56.7% cases and 57.5% controls have cousin marriages. While 43.3% and 42.5% respectively cases and controls were not with cousin marriages. Hence, for cousin marriages, controls were found greater than the cases (Figure 3.23).

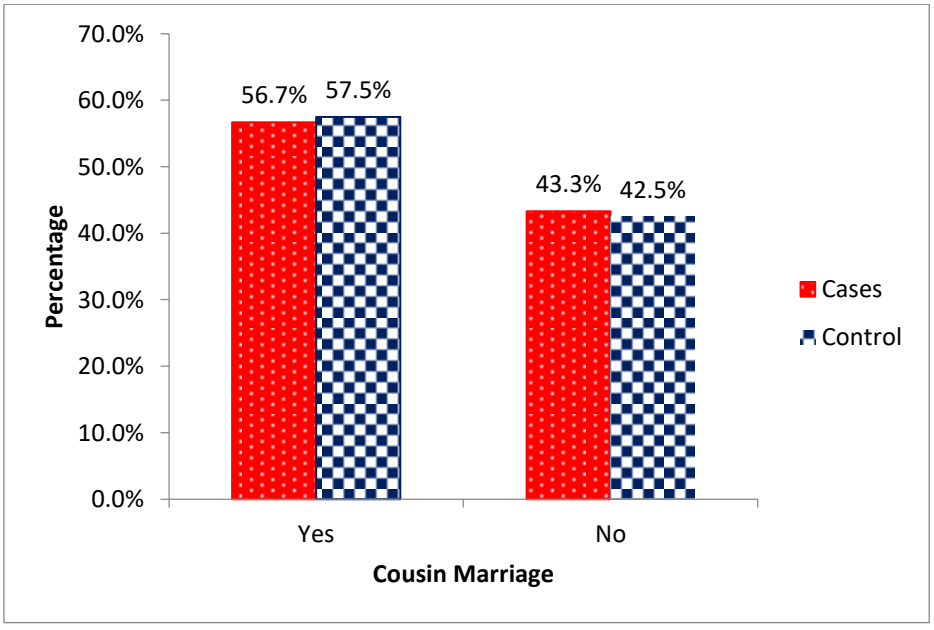

Figure 3.23 Percentage of cases and controls categorized on the basis of cousin marriage

3.5.14 Infertility Treatment

Percentage of cases and controls were investigated in patients for infertility treatment and it is found that 13.3% cases in comparison to controls i.e. 7.9%. Whereas, patients without infertility treatments were 86.7% cases and 92.1% controls. With infertility treatment, cases were reported higher than controls (Figure 3.24).

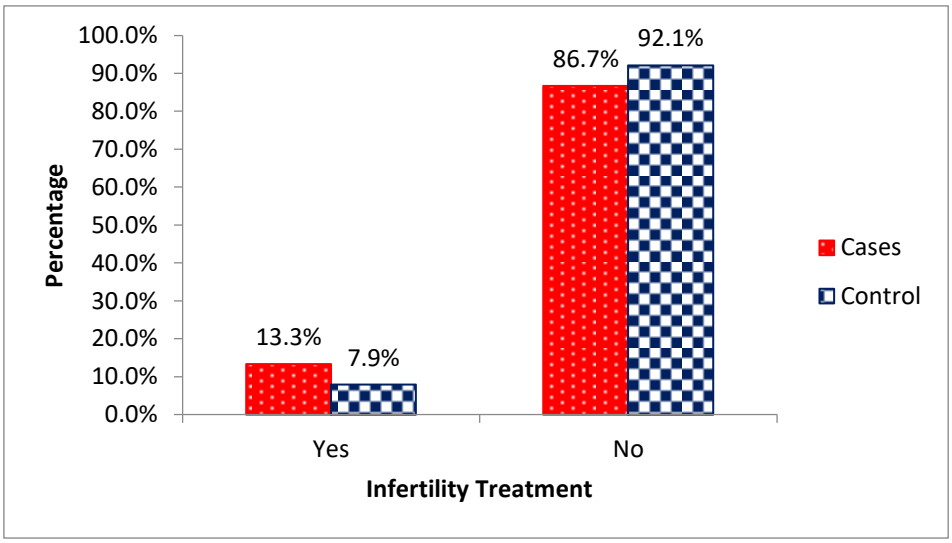

Figure 3.24 Percentage of cases and controls categorized on the basis of infertility treatment

3.5.15 Previous Fetal Complications

Patient's percentage of cases and controls were subdivided on the basis of previous fetal complications. Patients with previous fetal complications have greater percentage of cases than controls i.e. 5% cases and 2.5% controls. While 95% cases and 97.5% control did not have previous history of fetal complications (Figure 3.25).

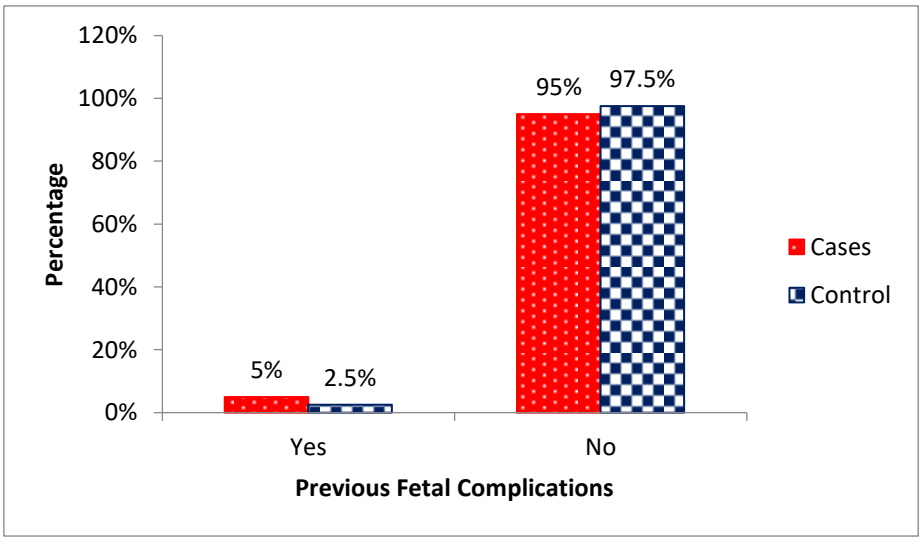

Figure 3.25 Percentage of cases and controls categorized on the basis of previously effected child

3.5.16 Current Mode of Delivery

Current modes of delivery were checked in patients; cesarean cases were found greater than controls and vaginal controls were observed higher than cases. About 85% cesarean were found in cases but 75.8% in controls. While 24.2% with vaginal delivery mode was found in controls and 15% in cases as shown in the following figure 3.26.

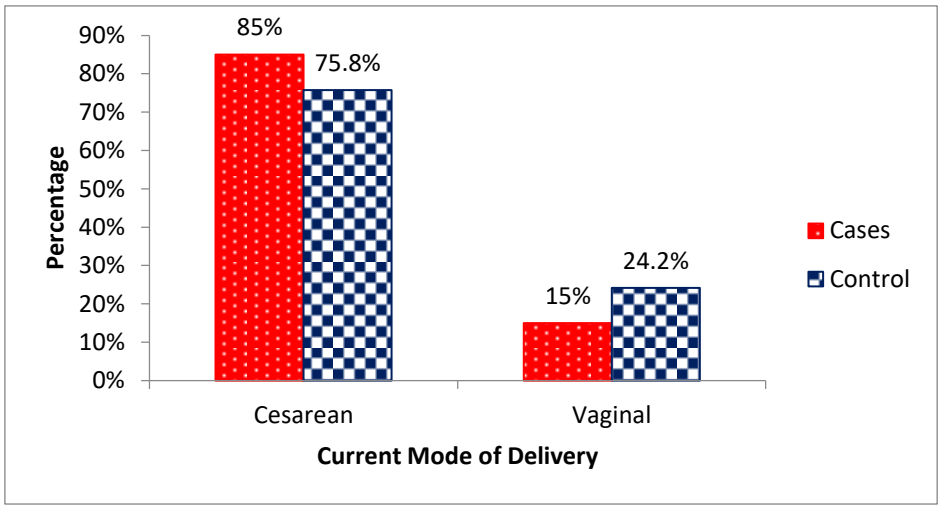

Figure 3.26 Percentage of cases and controls categorized on the basis of current delivery mode

3.5.17 Previous Mode of Delivery

Patients were investigated for mode of delivery in previous pregnancy including cesarean and vaginal. In cesarean, controls were found higher than cases as cases were 28.3% and 44.6% were controls. In vaginal, cases were found higher than control as 36.7% were cases and 26.3% were controls. While 35% cases and 29.2% controls were reported with their first pregnancy (Figure 3.27).

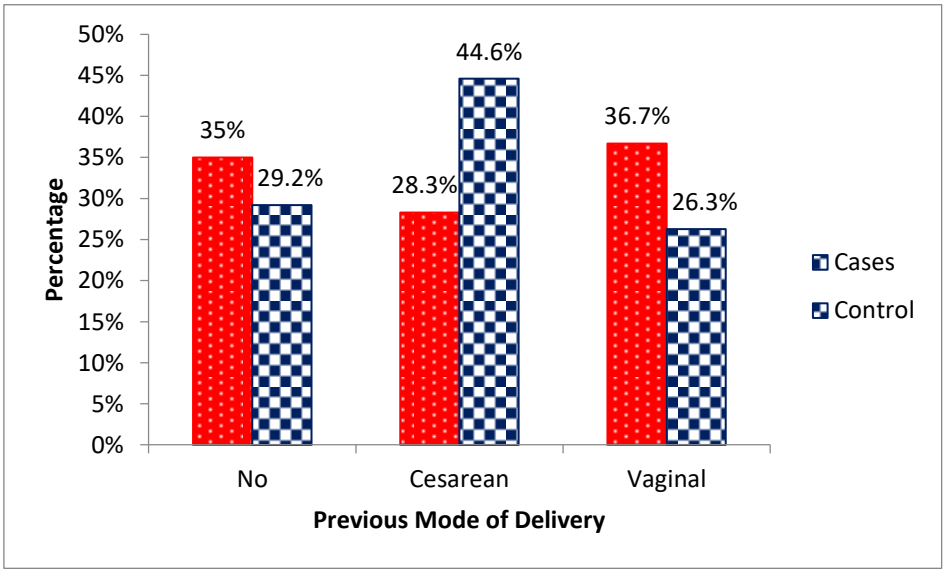

Figure 3.27 Percentage of cases and controls categorized on the basis of previous delivery mode

3.5.18 Stillbirth in Current Pregnancy

Patients with cases and control were investigated for stillbirth in current pregnancy. Cases 13.3% with stillbirth were found higher than controls i.e. 2.10%. While with no stillbirth in current pregnancy, patient with control 97.90% were greater in comparison to cases i.e. 86.70% (Figure 3.28).

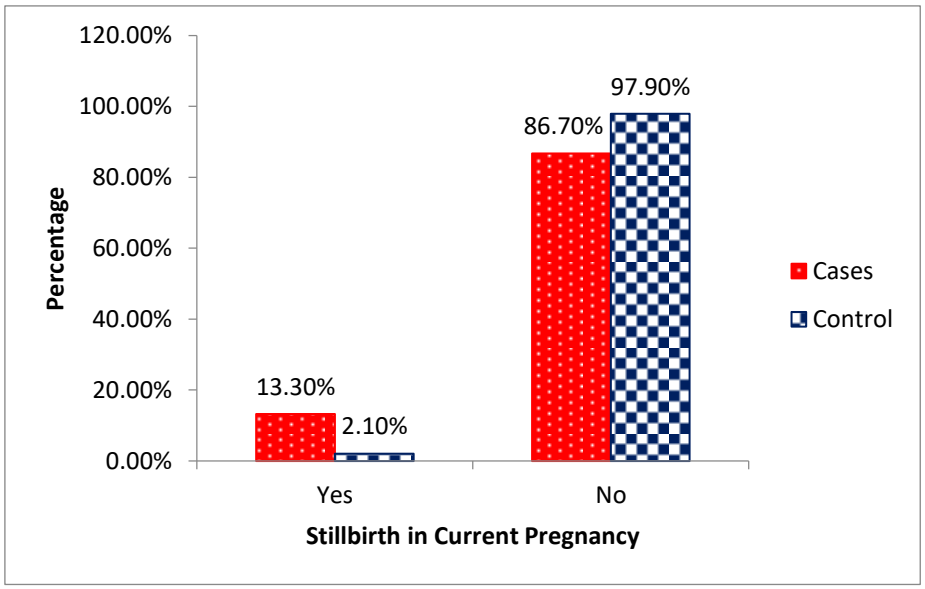

Figure 3.28 Percentage of cases and controls categorized on the basis of stillbirth

3.5.19 Neonatal Morbidity

Patients with neonatal morbidity have greater cases than controls as cases were 8.3% and 3.3% controls were observed with neonatal morbidity as shown in figure 3.29. (Table 3.6)

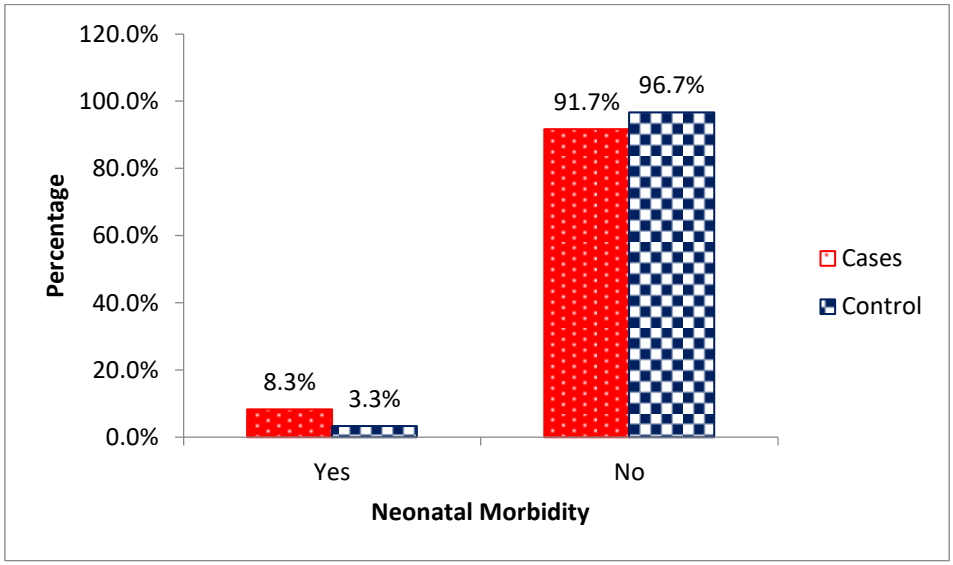

Figure 3.29 Percentage of cases and controls categorized on the basis of Neonatal Morbidity

3.5.20 Deceased Child History of Previous Pregnancies

Patients were investigated for deceased child history. Cases were surprisingly found to have less percentage than the controls. Cases were found as 1.7% with deceased history of child, while controls were 8.8%. Without the history of deceased child, 98.3% cases were found higher than controls 91.3% (Figure 3.30).

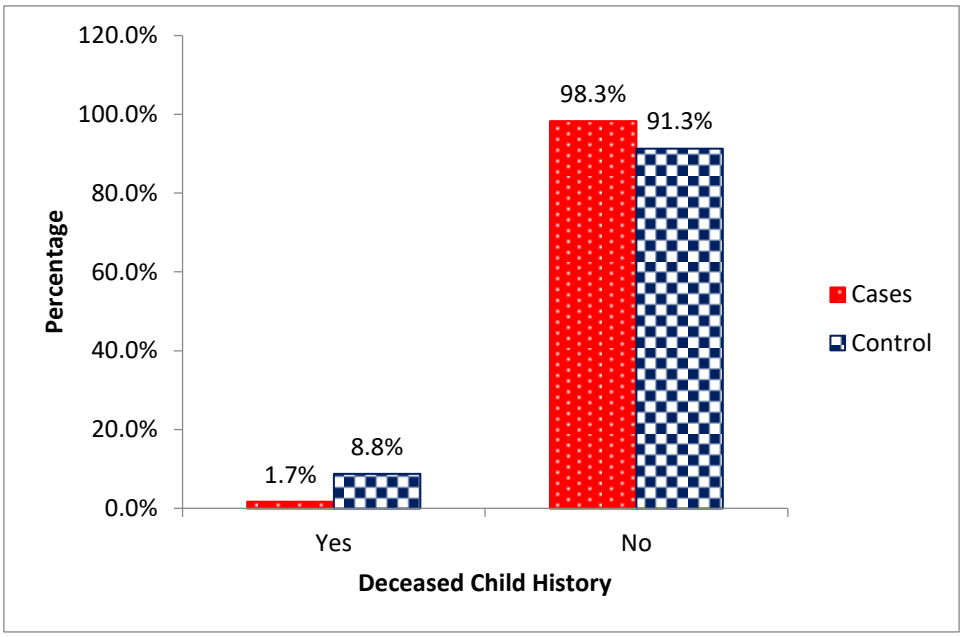

Figure 3.30 Percentage of cases and controls categorized on the basis of deceased child history

3.5.21 Gestational Diabetes

Patients were checked for gestational diabetes. 16.7% cases and 8.3% controls were observed with gestational diabetes. Patients with gestational diabetes have greater percentage of cases than controls (Figure 3.31).

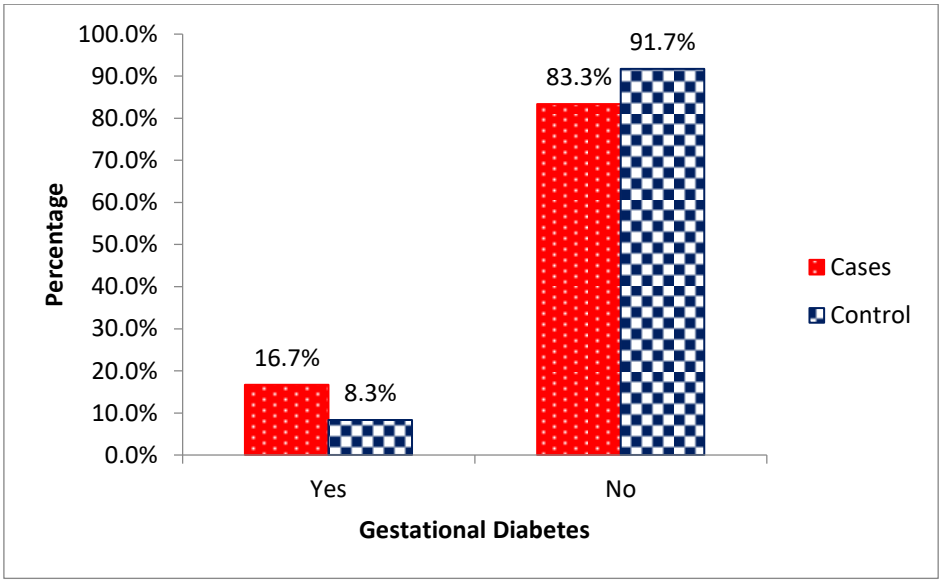

Figure 3.31 Percentage of cases and controls categorized on the basis of gestational diabetes

3.5.22 Pre-Gestational Diabetes

Patients were checked for pre-gestational diabetes. 1.7% cases and 0.8% controls were observed with pre-gestational diabetes. Patients with pre-gestational diabetes have greater percentage of cases than controls (Figure 3.32).

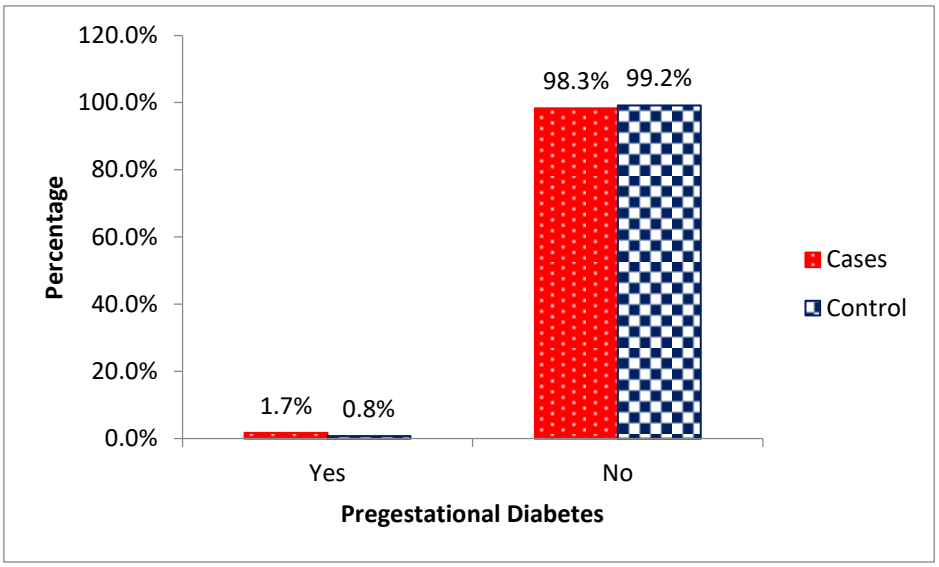

Figure 3.32 Percentage of cases and controls categorized on the basis of pre-gestational diabetes

3.5.23 Previous History of PIH

Patients were categorized into cases and controls with or without previous history of PIH. 13.3% cases and 8.8% controls were observed with previous history of PIH. Patients with previous history of PIH have greater percentage of cases than controls. 35% cases and 24.2% controls were primiparous in our study so they don't have previous history of PIH (Figure 3.33).

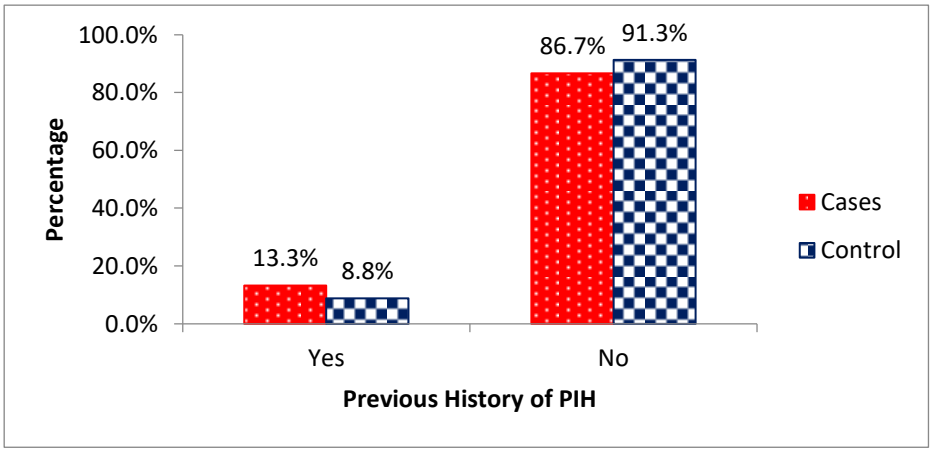

Figure 3.33 Percentage of cases and controls categorized on the basis of previous history of PIH

3.5.24 Blood Group

Patients were subdivided into cases and controls with different types of blood group including A +ive, A -ive, B +ive, B -ive, AB +ive, O –ive and O +ive. Blood group A +ive have 16.7% cases, A –ive with 1.7%, B +ive with 10%, B –ive have 0%, O +ive with 45%. While, O –ive and AB +ive with 3.3% cases. Patients with blood group O +ive were observed with highest percentage of PE cases but least with B –ive (Figure 3.34).

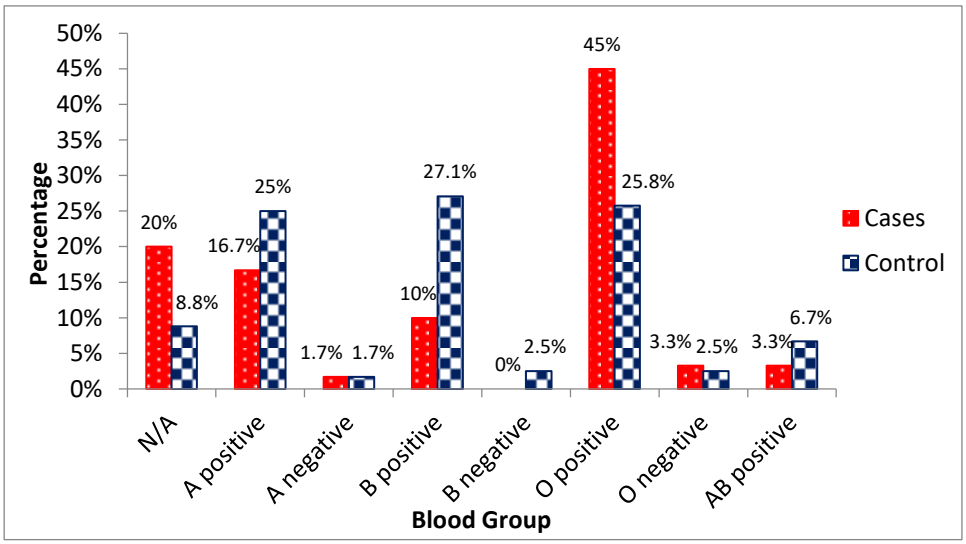

Figure 3.34 Percentage of cases and controls categorized on the basis of blood group

3.5.25 Infant Sex

Patients were divided into cases and controls, which are further subdivided on the basis of infant sex including boy, girl, both girls and both boys. Boy has 45% cases, while girl with 51.7% cases. On the other hand, twin pregnancies with both boys have 1.7% cases and both girls with zero percentage. Girls were found to have highest percentage of cases but both girls with least (Figure 3.35).

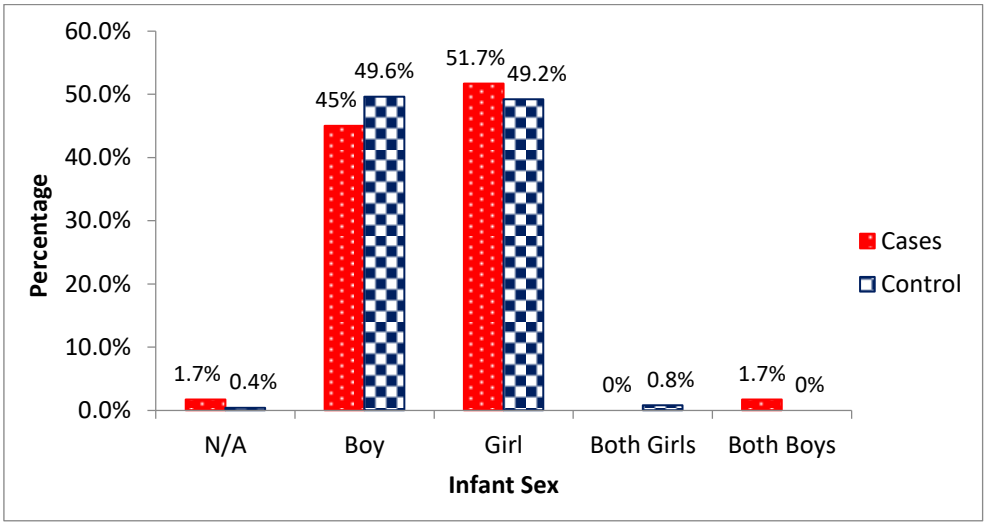

Figure 3.35 Percentage of cases and controls categorized on the basis of infant sex

3.5.26 Infant Weight

Patients were categorized into cases and controls, which are further subdivided on the basis of infant weight including ≤2 kg, 2.1-3.5 kg and ≥3.6 kg. Infants with weight ranges 2.1-3.5 kg were observed to have highest percentages of cases as compared to other weight groups. Weight group ≤2 kg was lying in 5% cases, while group with weight ≥3.6 kg has 1.7% cases. Cases were found to have low infant weight (Figure 3.36).

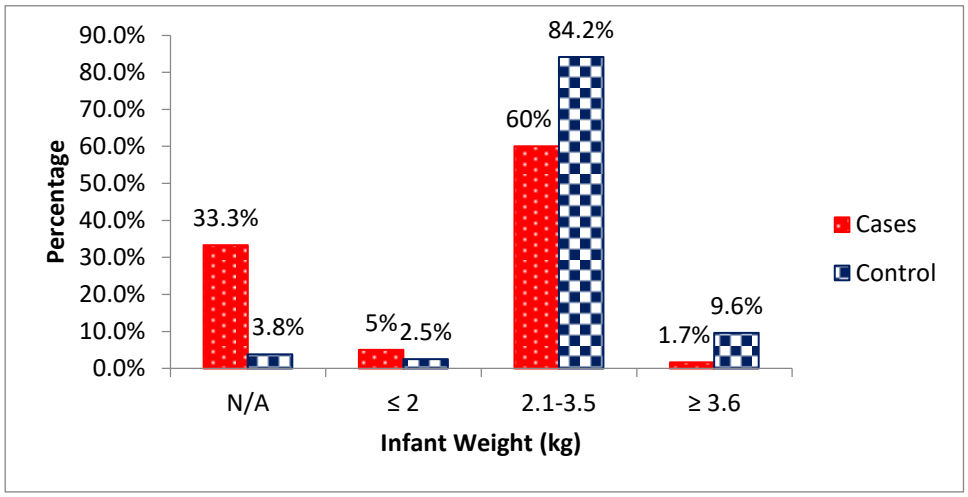

Figure 3.36 Percentage of cases and controls categorized on the basis of infant weight

3.5.27 Geographic Distribution of Participants

Study was conducted at CMH Muzaffarabad, therefore greatest percentages of cases were found from Muzaffarabad district as compared to other. Cases were relatively found to have 78.3%, 5%, 3.3%, 11.7% and 1.7% for district Muzaffarabad, Neelum, Bagh, Hattian Bala and others (Figure 3.37).

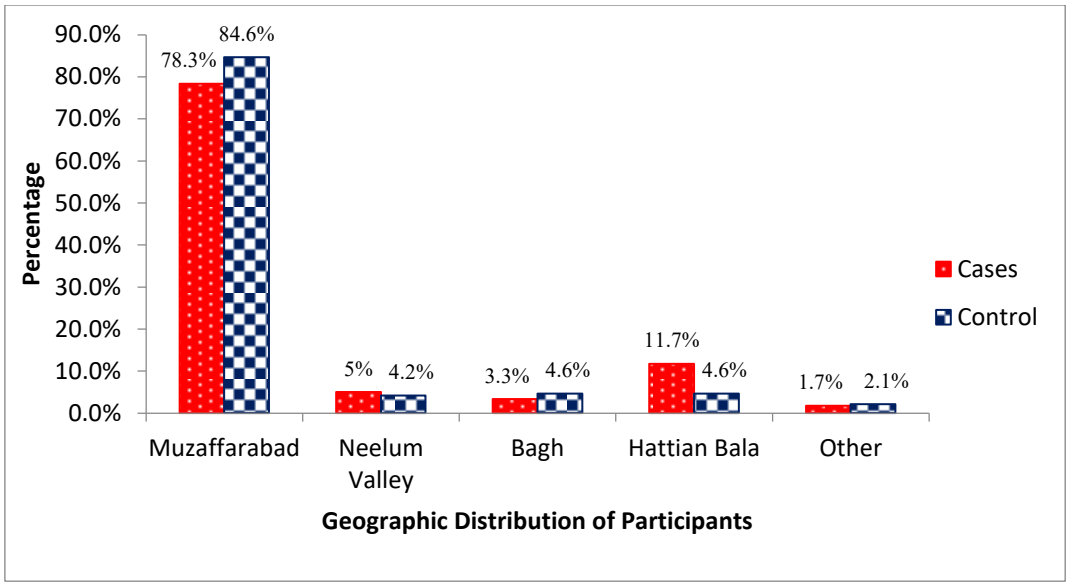

Figure 3.37 Percentage of cases and controls categorized on the basis of address

3.6 Results of Risk factor Analysis by cox regression model

Based on literature search, previously reported demographic and medical related risk factors/exposures were investigated in our study cases using cox regression model. Cox regression model used to analyze risk factors showed that parity, miscarriage, supplements intake during pregnancy and family history of hypertension were significantly associated with preeclampsia (P<0.05) in our study as shown in Table 3.4. However, multiparity and proper intake of supplements were found to be responsible for lowering the

risk of PE (OR<1, 95%CI) while the other risk factors i.e. history of miscarriage and family history of disease were found to increase risk (OR>1, 95%Cl) as shown in Table 3.4.

It was hypothesized that primiparous women have more risk of preeclampsia. In our study, *P* = 0.026 for parity showed that there was a highly significant association between parity and preeclampsia. However, multiparity with OR = 0.370 (0.154-0.886) was found linked to lower the risk of PE. Intake of supplements with OR = 0.483 (0.229-1.018) and *P* = (0.054) shows significant relation with PE. Previous history of miscarriage with OR = 1.740 (1.083-2.795) and *P* = 0.022 was found to be significantly associated with PE in current pregnancy. Family history of hypertension with *P* < 0.0001, indicated that family history of hypertension have very highly significant relation with PE, while its OR = 9.235 (4.523-18.858) suggested that women with family history of hypertension will be at increased risk of preeclampsia.

Among reproductive risk factors gestational age and age at marriage while among medical risk factors chronic hypertension, previous history of PIH, pregestational diabetes, fertility treatment, GDM in previous pregnancy, previous fetal complications, stillbirth in current pregnancy, deceased child history of previous pregnancy and neonatal morbidity all were found not linked to disease risk. However all lifestyle risk factors i.e. education, occupation, consanguinity, smoking during pregnancy and all anthropometric risk factors that includes BMI, patient blood group, infant sex, singlets/twins fetus and infant weight were found not linked to PE risk.

Table 3.4 Analysis of different risk factors for preeclampsia onset by cox regression model

	Characteristics	Regression Coefficient(ß)	*P*-value	OR (Exp ß)	95% CI of expected B	
					Lower	Upper
Reproductive risk factors	**Gestational Age**	-0.200	0.118^{NS}	0.819	0.637	1.052
	≤30 30-38 ≥39					
	Parity	-0.995	0.026**	0.370	0.154	0.886
	Primiparity Multiparity Grandparity					
	Miscarriage	0.554	0.022**	1.740	1.083	2.795
	Yes No					
	Age At Marriage	0.555	0.089^{NS}	1.742	0.918	3.304

	≤ 20 21-28 ≥ 29					
Medical Related Risk Factors	**Chronic Hypertension**	0.140	0.822^{NS}	1.150	0.339	3.899
	Yes No					
	History Of PIH	-0.190	0.691^{NS}	0.827	0.323	2.115
	Yes No					
	Pre gestational Diabetes	0.730	0.519^{NS}	2.074	0.226	19.032
	Yes No					
	Supplements Intake	-0.728	0.054*	0.483	0.229	1.018
	Yes No					
	Fertility Treatment	0.138	0.782^{NS}	1.148	0.431	3.057
	Yes No					
	Family History	2.223	< 0.0001***	9.235	4.523	18.858
	Yes No					
	GDM in Previous Pregnancy	-0.307	0.522^{NS}	0.736	0.288	1.883
	Yes No					
	Previous Fetal Complications	1.529	0.067^{NS}	4.612	0.899	23.671
	Yes No					
	Stillbirth in Current Pregnancy	0.453	0.545^{NS}	1.573	0.363	6.829
	Yes No					
	Deceased Child History	-0.474	0.663^{NS}	0.622	0.074	5.246
	Yes No					
	Neonatal Morbidity [a]	-0.071	0.932^{NS}	0.932	0.182	4.757
	Yes No					
Lifestyle Risk Factors	**Education**	-0.184	0.304^{NS}	0.832	0.585	1.182
	Uneducated Primary Secondary Higher Education					

	Occupation	0.329	0.482^{NS}	1.390	0.555	3.483
	Student Housewife Working					
	Consanguinity	-0.366	0.249^{NS}	0.693	0.372	1.293
	Yes No					
	Smoking during pregnancy	0.562	0.591^{NS}	1.754	0.226	13.632
	Yes No					
Anthropometric Risk Factors	**BMI**	0.037	0.206^{NS}	1.038	0.980	1.099
	< 18.5 18.5-24.9 25-29.9 > 30					
	Blood Group	0.010	0.885^{NS}	1.011	0.877	1.165
	N/A A positive A negative B positive B negative O positive O negative AB positive					
	Infant Sex	0.144	0.603^{NS}	1.155	0.672	1.986
	N/A Boy Girl Both Girls Both Boys					
	Infant Weight(kg)	-0.042	0.761^{NS}	.959	0.731	1.258
	N/A ≤ 2 2.1-3.5 ≥ 3.6					
	Singlets/Twins Fetus	-0.368	0.670^{NS}	0.692	0.127	3.759
	Single Twins					

CI: confidence intervals

OR: odds ratio

^{NS}: Non-Significant

*: Significant association

**: Highly Significant association

***: Very highly significant association

[a]: Includes any of the following: respiratory distress, dehydration, blood infection, premature fetus and aspired meconium.

Section B-Results of Molecular and Genetic Studies

Previously variants of different genes e.g. *CYP11B2*, *MTHFR*, *eNOS*, *CXCR2*, *STOX1* and *VDR* etc. are reported to be linked with increased risk of PE in women of various geographic background. However literature search showed that *VDR* polymorphisms were never studied in PE affected women from AJK and Pakistan. *VDR* gene *FokI*, *BsmI*, *TaqI* and *ApaI* polymorphisms are reported to be linked with increased risk of PE (Farajian-Mashhadi *et al.*, 2020; Rezavand *et al.*, 2019) and other pregnancy related disorders including GDM (Liu, 2021), type1 diabetes in the offspring (Miettinen *et al.*, 2015), preterm birth (Rosenfeld *et al.*, 2017), and respiratory distress syndrome (RDS) in preterm infants (Ustun *et al.*, 2020). Based on this background, we selected *FokI* polymorphism of exon 2 of *VDR* gene for molecular analysis. Previously *FokI* polymorphism rs2228570 (c.2T>C) has been reported to be linked with PE in Iran (Farajian-Mashhadi *et al.*, 2020) and China (Zhan *et al.*, 2015).

In this study, a total of 60 women were presented with PE at CMH hospital Muzaffarabad within April to July 2021. However, only 52 women agreed to participate in molecular analysis and their blood samples were collected. Therefore, DNA of 52 samples was extracted. Extracted DNA was used to amplify the exon 2 of *VDR* gene by using primer described in table 2.3 to screen the minor allele *FokI* polymorphism rs2228570. A restriction fragment length polymorphism (RFLP) technique was used to confirm the T>C polymorphism in the population. *FokI* Fast Digest restriction enzyme (ThermoFisher Scientific Company) cuts a wild type sequence into two bands of sizes 197 and 62bp. However, if the target sequence contains T>C polymorphism, the enzyme do not cleave and a results in a single band.

PCR products after restriction enzyme digestion were loaded on 2.5% gel and genotypes were predicted on the basis of bands shown on agarose gel as enlisted in table 3.5. PCR was performed on 52 collected samples of PE patients, but only 40 samples were amplified and shows band on agarose gel, while 12 (PE11, PE17, PE19, PE21, P24, PE25, PE27, PE28, PE29, PE34, PE38, PE42) shows no band. In order to check *VDR FokI* polymorphism (T>C), RFLP was executed on 40 amplified samples, out of which 26 samples cleaved into two bands of 197 and 62 bp long, whose genotype is wild type i.e. TT, and 6 samples of genotype TC showed heterozygosity and produced three bands of 259, 197 and 62 bp long on agarose gel, while 8 samples with homozygous CC genotype don‘t gets cut at all.

Table 3.5 Predicted genotypes of cases on the basis of RFLP bands on gel

Sr. No.	Patient ID	RFLP bands on gel			Predicted Genotype
		259bp	197bp	62bp	
1	**PE1**	No	✓	✓	TT
2	**PE2**	✓	✓	✓	TC
3	**PE 3**	No	✓	✓	TT
4	**PE 4**	No	✓	✓	TT
5	**PE 5**	✓	✓	✓	TC
6	**PE 6**	✓	No	No	CC
7	**PE 7**	✓	No	No	CC
8	**PE 8**	✓	No	No	CC
9	**PE 9**	✓	No	No	CC
10	**PE 10**	✓	No	No	CC
11	**PE 12**	✓	✓	✓	TC
12	**PE 13**	No	✓	✓	TT
13	**PE 14**	No	✓	✓	TT
14	**PE 15**	No	✓	✓	TT
15	**PE 16**	No	✓	✓	TT
16	**PE 18**	No	✓	✓	TT
17	**PE 20**	No	✓	✓	TT
18	**PE 22**	No	✓	✓	TT
19	**PE 23**	No	✓	✓	TT
20	**PE 26**	✓	✓	✓	TC
21	**PE 30**	✓	✓	✓	TC
22	**PE 31**	No	✓	✓	TT
23	**PE 32**	✓	No	No	CC
24	**PE 33**	No	✓	✓	TT
25	**PE 35**	No	✓	✓	TT
26	**PE 36**	No	✓	✓	TT
27	**PE 37**	No	✓	✓	TT
28	**PE 39**	No	✓	✓	TT
29	**PE 40**	✓	No	No	CC
30	**PE 41**	No	✓	✓	TT
31	**PE 43**	No	✓	✓	TT
32	**PE 44**	No	✓	✓	TT
33	**PE 45**	No	✓	✓	TT
34	**PE 46**	No	✓	✓	TT
35	**PE 47**	✓	No	No	CC
36	**PE 48**	✓	✓	✓	TC
37	**PE 49**	No	✓	✓	TT
38	**PE 50**	No	✓	✓	TT
39	**PE 51**	No	✓	✓	TT
40	**PE 52**	No	✓	✓	TT

A 100bp DNA ladder was run along with the samples to determine the band size as shown in figure 3.38. We loaded the DNA ladder in the first well and the PCR product which is not digested by enzyme served as a control group in the second well to see the difference in band size before and after restriction enzyme digestion. PE13, PE14, PE22, PE23, PE35, PE44 and PE49 were cleaved by restriction enzyme and showed two bands on gel of 197 and 62bp which were wild type of genotype TT. PE2 and PE12 were cut by enzyme and produced three bands of 259, 197 and 62 bp which represents heterozygosity with genotype TC. While, PE1 and PE8 were not digested by the enzyme due to presence

of *FokI* polymorphism in the restriction site of enzyme which represents genotype CC. (Figure 3.38)

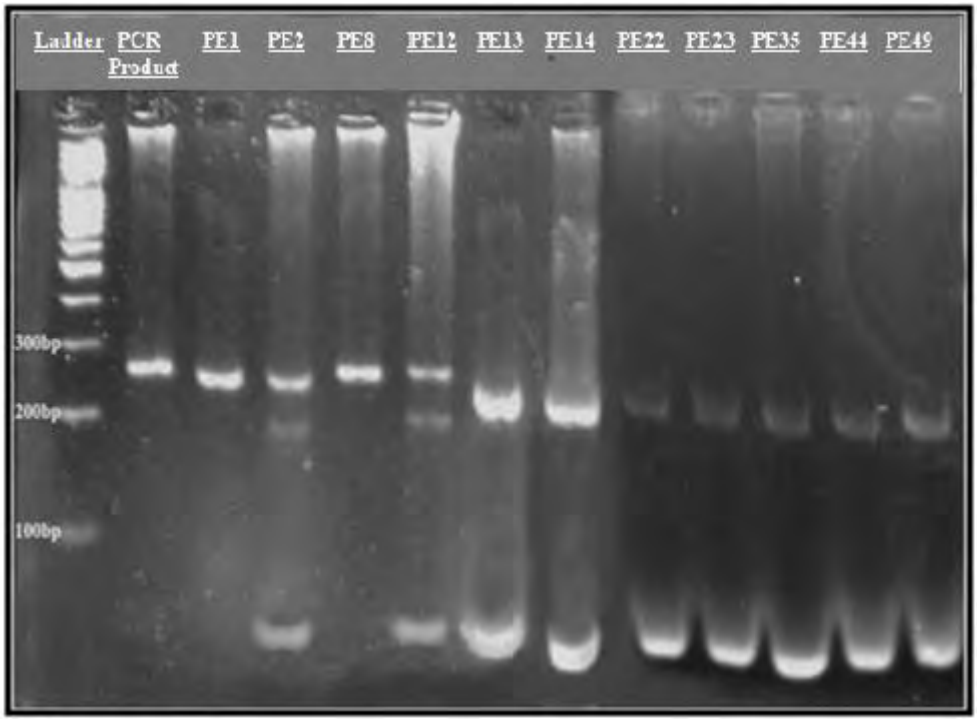

Figure 3.38 Agarose gel picture after restriction digestion with *FokI* endonuclease of some selected PE cases

PCR was also performed on 16 collected samples of controls, but only 9 samples were amplified and showed band on 2% agarose gel, while 7 did not show band. RFLP was performed on these 9 amplified samples to check the *FokI* polymorphism of *VDR* gene, polymorphism was not found in any of the control samples as all the controls were wild type i.e. TT genotype, produced two bands of 197 and 62 bp long.

3.7 Percentage Distribution of Genotypes and alleles among Cases and Controls

The frequency and percentages of *FokI* genotypes and alleles among 40 PE patients and 9 controls are enlisted in table 3.6. Cases and controls were subdivided into three genotypes i.e. TT, TC and CC. TT showed two bands on gel indicating no polymorphism, TC showed three bands on gel which is the indication of heterozygosity with one wild type allele and other variant. CC genotype revealed one band only showing homozygous polymorphism. Genotype TT, TC and CC were found 26 (65%), 6 (15%) and 8 (20%) respectively in cases, whereas all 9 controls (100%) were wild type i.e. TT genotype. Allele T and C were found 58 (72.5%), 22 (27.5%) in cases respectively, while 18 T alleles

(100%) and no C allele were found in controls. It depicts that polymorphism was not present in controls.

Table 3.6 Distribution of *VDR FokI* genotypes a well as alleles in PE patients and controls

Gene *VDR* rs2228570 (T>C)	**Cases** N=40 N,%	**Controls** N=9 N,%
***FokI* genotypes**		
TT	26 (65%)	9 (100%)
TC	6 (15%)	0 (0%)
CC	8 (20%)	0 (0%)
TT + TC	32 (80%)	9 (100%)
Alleles		
T	58 (72.5%)	18 (100%)
C	22 (27.5%)	0 (0%)

N: number/frequency

Percentage distribution of genotypes among cases and controls are displayed in figure 3.39. Genotype TT, TC and CC were found 65%, 15% and 20% respectively in cases. All controls (100%) were found to have wild type TT genotype. Genotypes in cases were found to follow the order as TT>CC>TC which is shown in the figure 3.39.

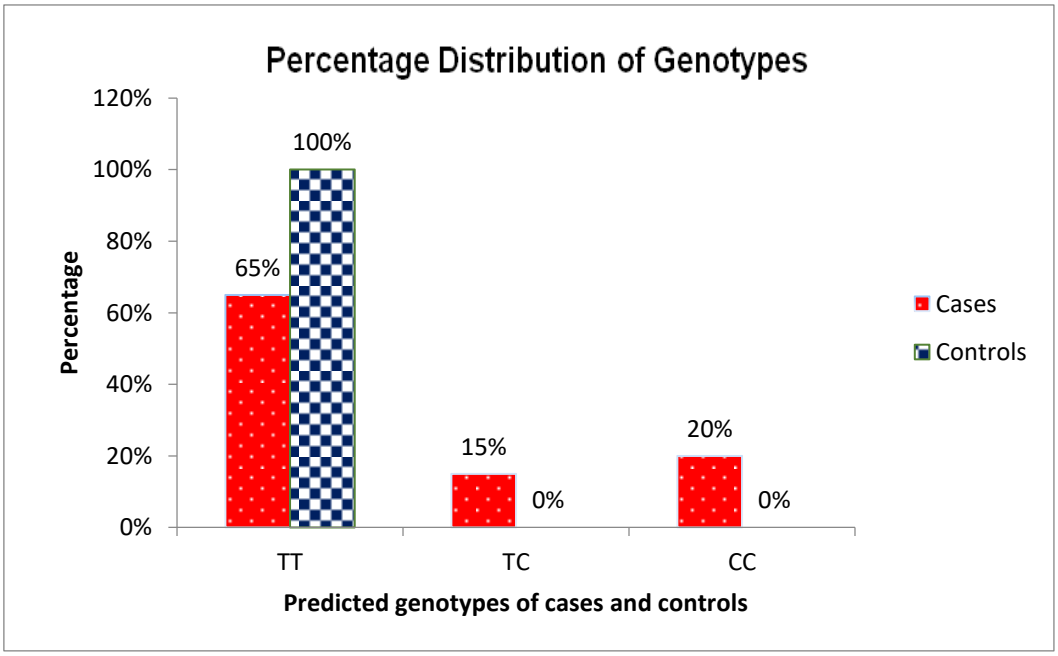

Figure 3.39 Representation of percentage distributions of predicted genotypes of cases and controls

Percentage distribution of predicted alleles among cases and controls are depicted in figure 3.40. Allele T was found 72.5% and 100% in cases and controls respectively. Allele C was found 27.5% in cases while 0% in controls.

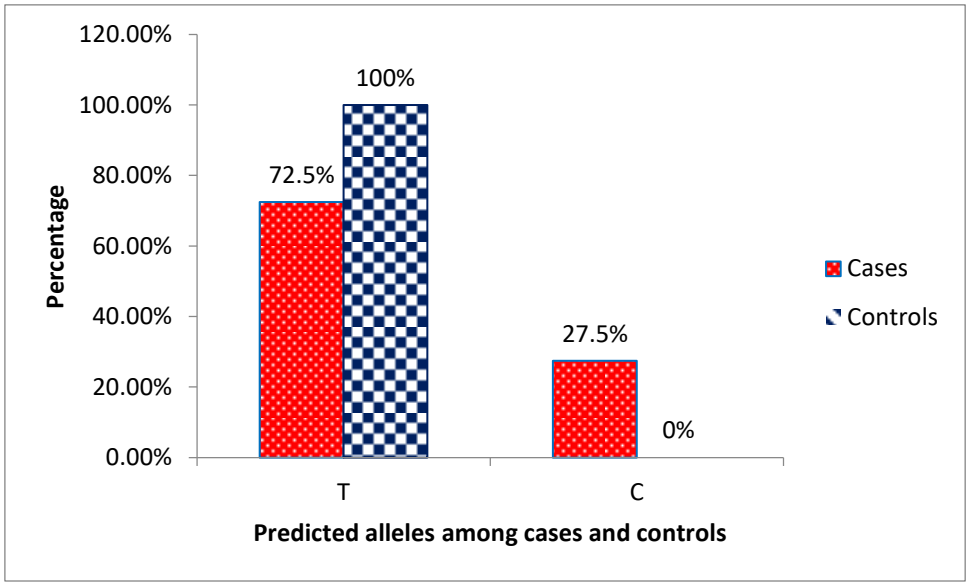

Figure 3.40 Representation of percentage distribution of predicted alleles among cases and controls

DISCUSSION

Preeclampsia is the most common disorder among pregnant women worldwide. It affects about 4.6% of the pregnancies worldwide (Abalos *et al.*, 2013). It is linked with 1 maternal death per 100,000 live births in the developed countries and results in more than 60,000 maternal deaths annually (Duhig *et al.*, 2018; Gao *et al.*, 2021). Preeclampsia and eclampsia are responsible for about a third of a million deaths in low and middle income families and more than six million perinatal deaths (Bilano *et al.*, 2014). According to a systemic analysis of global mortality, Pakistan has the third-highest burden of fetal, child, and maternal mortality (Bhutta *et al.*, 2013). Pregnant women without proteinuria were diagnosed by the hypertension along with renal insufficiency, thrombocytopenia, pulmonary edema, impaired liver function and cerebral or visual disturbances (Obstetricians & Gynecologists, 2013). The etiology of PE still remains unknown therefore, screening tools and preventive measures for PE are lacking. Its treatment involves the management of adverse symptoms and delivery which are the only ultimate cure (Norwitz *et al.*, 2009). In present study, clinical as well as demographic characters and PE risk factors were evaluated. Moreover, some lifestyle factors / medical exposures and reproductive factors were also analyzed using concept of age matched controls by cox regression model and results were described in terms of odd ratios.

The age distribution of PE patients as well as controls represents that 10% patients were from the age group ≤ 21, 43.3% from the 22-29 age group, 38.3% lied in the range of 30-37 and 8.3% were lie in the age group ≥ 38. Mean age recorded for cases and controls was 29 years in the present study. Studies reported that women older than 35 years have 4-5 fold high risk of suffering PE compared to women of 25-29 years age (Li *et al.*, 2018; Tyas *et al.*, 2019) whereas, in our study, most of the PE patients were 22-29 years old.

Results of risk factors analysis by cox hazardous regression model showed that parity (P=0.026), history of miscarriage (P=0.022), supplements intake during pregnancy (P=0.054) and family history of hypertension (P<0.0001) were significantly associated with preeclampsia in this study. Among these risk factors multiparity and proper supplements intake during pregnancy were found to be responsible for decreasing the risk of PE (O.R<1, 95%CI) while history of miscarriage and family history of hypertension were found to increase risk (O.R>1, 95%Cl). Similar results were reported in many previous studies conducted by Shamsi *et al.*, You *et al.*, English *et al.*, and Pipkin *et al.* who reported that primiparity, proper intake of supplements during pregnancy and a family

history of hypertension are significantly associated with the PE (English *et al.*, 2015; Pipkin, 2001; Shamsi *et al.*, 2010; You *et al.*, 2018). Sibai *et al*. observed no significant association between history of abortion or miscarriage with risk of PE that is contradictory to our findings (Sibai *et al.*, 1997). This inconsistency in results might be due to varied study area, small sample size and less and varied time duration of our study.

In our results, chronic hypertension, BMI, GDM, history of diabetes mellitus, history of PIH, infertility treatment, smoking during pregnancy, infant sex and weight showed no significant association ($P>0.05$) with PE risk. Several previous studies by You *et al*., Quan *et a*l., Li *et al*., and Lisonkova *et al*. showed significant association between BMI, infertility treatment, preexisting hypertension, pregestational diabetes, male fetus, very low birth weight, previous PIH, GDM and risk of PE (Li *et al.*, 2018; Lisonkova & Joseph, 2013; Quan *et al.*, 2018; You *et al.*, 2018). However, Lisonkova *et al*. reported that women who smoked during pregnancy had a lower risk of late onset PE (Lisonkova & Joseph, 2013).

In this study, different clinical parameters of PE affected women were also analyzed. Out of which mean serum values of creatinine, bilirubin and urea were significantly raised in cases compared to controls ($p<0.0001$). These results are similar to the studies performed on India and Zimbabwe populations that reported an elevation in the average values of serum creatinine and urea in PE patients (Makuyana *et al.*, 2002; Manjareeka & Nanda, 2013; Monteiro *et al.*, 2013; Vyakaranam *et al.*, 2015). Our findings were inconsistent with previous study Müller-Deile *et al*. which found that blood creatinine and urea nitrogen levels in PE patients are similar to the levels found in non-pregnant female because of decreased renal plasma flow as well as glomerular filteration rate in PE patients, although normal pregnancy is characterized by increased renal plasma flow as well as glomerular filteration rate (Davison & Dunlop, 1980; Müller-Deile & Schiffer, 2014; Sims & Krantz, 1958). The elevation in the serum creatinine level might be due to reduce urinary clearance secondary to increased reabsorption and decreased glomerular filteration rate (Jeyabalan & Conrad, 2007). Mean value of serum bilirubin was found significantly increased in cases than the controls that are consistent with the previous studies (Jaleel *et al.*, 1999; Malvino *et al.*, 2005). They reported that serum bilirubin level is higher in PE and HELLP syndrome patients in comparison to normotensive controls. A significant elevation ($p<0.0001$) of alkaline phosphatase (ALP), alanine transaminase (ALT), urinary protein, systolic blood pressure (SBP) and diastolic blood pressure (DBP) was observed in PE patients during this study. Similarly, many researchers also found

significantly higher levels of ALP, ALT, urinary protein, systolic and diastolic blood pressure in patients which is consistent with our results (Ekun *et al.*, 2018; Knapen *et al.*, 1998; Kurt *et al.*, 2015; Li *et al.*, 2018; Makuyana *et al.*, 2002; Malvino *et al.*, 2005; Munazza *et al.*, 2011). However, several studies found no significant elevation of ALP and ALT in PE patients which is not in line with our findings (He *et al.*, 1995). This difference in result might be due to varied sample size and study population.

In this study, significantly higher blood hemoglobin (Hb) ($p<0.0001$) and platelets count ($p<0.05$) were observed in controls as compared to PE patients but mean value of platelet count falls in a normal range. Our findings are consistent with studies of many previous researchers who found that mean values of blood Hb and platelet count were higher in normotensive controls compared to PE patients (Hayashi *et al.*, 1999; Kirbas *et al.*, 2015; Li *et al.*, 2018; Singer *et al.*, 1986). Our finding is contradictory to Kurt *et al*. who reported that level of platelet count and blood Hb in PE patients and controls were similar (Kurt *et al.*, 2015).

VDR gene polymorphisms were reported to be linked with increased risk of PE (Farajian-Mashhadi *et al.*, 2020; Rezavand *et al.*, 2019; Zhan *et al.*, 2015). Bodnar *et al*. reported that pregnant women with vitamin D deficiency were at increased risk of severe PE (Bodnar *et al.*, 2014). To the best of knowledge, it is the first study of *VDR* polymorphisms among Kashmiri pregnant women affected with preeclampsia, as *VDR* polymorphisms were never studied in PE affected women from AJK and Pakistan.

In current study, we examined the sequence variant of initiation codon of exon 2 of *VDR* gene (*Fok*I polymorphism) in PE women for its contributing role. We found that pregnant women carrying ‗C' allele of *Fok*I polymorphism of *VDR* gene were at high risk of PE. Rezavand *et al*. reported that *VDR Fok*I polymorphism was associated with 1.72-fold increased risk of PE (Rezavand *et al.*, 2019).

To check the *VDR* polymorphism in PE patients, molecular analysis was performed. RFLP was used to detect the *VDR FokI* polymorphism because of its high reliability, marker specificity, high reproducibility and co-dominance that enabled us to discriminate heterozygotes from the homozygotes as done previously by many researchers (Farajian-Mashhadi *et al.*, 2020; Ghorbani *et al.*, 2021; Magiełda-Stola *et al.*, 2021; Rezavand *et al.*, 2019; Swapna *et al.*, 2011). Out of 52 PE patients' samples, only 40 samples were amplified by PCR and showed band on agarose gel, whereas 12 samples did not give results as DNA was manually extracted so there are chances of presence of

impurities in the DNA sample and less DNA concentration as well. These 40 amplified samples were further subjected to RFLP to check the *VDR FokI* polymorphism. On the basis of number of bands obtained, three predicted genotypes were identified i.e. one wild type TT genotype that showed double bands on gel as restriction enzyme cut the DNA into two fragments of 197 and 62bp respectively, second CC genotype that showed polymorphism as only one band of 259bp which indicates that there is homozygous polymorphism in the restriction site of enzyme in DNA sample (Figure 3.38). While third TC genotype showed heterozygosity and produced three bands on gel as one allele was cut by restriction enzyme into two fragment of 197 and 62bp that is the indication of wild type T allele and second allele remains intact as restriction enzyme did not cleaved it due to polymorphism in its restriction site so the band of 259bp was observed (Figure 3.38).

Our results are consistent to the previous study by Rezavand *et al*. which showed that *VDR FokI* C allele is linked with risk of PE, where first translation initiation codon ATG is changed into ACG at the restriction site of *FokI* enzyme and translation begins at second ATG, producing a truncated protein with a reduction of three amino acids but with greater biological activity (Rezavand *et al.*, 2019). For the transactivation of 1, 25-(OH)2-D3 signal, C allele of *VDR FokI* polymorphism was found to be more effective in comparison to T allele (Huang *et al.*, 2006). Singla *et al*. reported that *VDR FokI* polymorphism is associated with reduced risk of hypertension that showed inconsistency with our findings (Singla, 2015). O'Callaghan *et al*. found the negative association between maternal 1, 25-(OH)2-D3 concentration and blood pressure as well as PE risk, but no association with the severity of PE (O'Callaghan & Kiely, 2018). While another study found no association between vitamin D fortification and PE risk as well as hypertension during pregnancy (Stougaard *et al.*, 2018).

In this study, percentage distribution of genotypes and alleles among cases and controls were also observed. 20% cases were found to have genotype CC and 15% with genotype TC, while 65% cases were wild type with TT genotype. Whereas all controls (100%) were wild type with TT genotype. A frequency of C allele was significantly increased in PE patients (27.5%) as compared to controls (0%) indicating that no _C' allele was present in controls which suggested that _C' allele *FokI* polymorphism is associated with PE risk. Caccamo *et al*. found that *VDR* FF/bB haplotype is associated with high blood pressure during pregnancy with two fold increased risk of gestational hypertension and 92% women with gestational hypertension that carries FF/bB haplotype were found

with insufficient vitamin D (Caccamo *et al.*, 2020). These findings suggest that *VDR FokI* polymorphism may influence the expression of *VDR* gene as well as the binding of 1, 25-(OH)2-D3 with *VDR* (Nunes *et al.*, 2016). Another study reported that there is an association between insufficiency of 1, 25-(OH)2-D3, as well as *VDR FokI* polymorphism and PE risk. They also found that women carrying *VDR FokI* polymorphism have increased systolic and diastolic blood pressure due to insufficient level of 1, 25-(OH)2-D3 (Zhan *et al.*, 2015).

Rezende *et al*. found a relationship between *FokI*, *ApaI*, *BsmI* polymorphism of *VDR* gene and risk of developing PE as well as gestational hypertension (Rezende *et al.*, 2012). The frequency of all these *VDR* polymorphisms was found similar in gestational hypertensive, preeclamptic and healthy normotensive pregnant females (Ghorbani *et al.*, 2021). Summarizing our findings it is suggested that there is a link between *FokI* polymorphism of *VDR* gene in our study and PE risk that is consistent to the previous study (Rezavand *et al.*, 2019). However, due to small sample size we would not confirm statistical significance of genotype data.

Conclusion

In conclusion, our study revealed that a family history of hypertension and previous miscarriage history were strongly associated with high risk of PE, while multiparity and proper supplements intake during pregnancy were strongly associated with decreased PE risk in Kashmiri women. Some clinical and pathological findings such as SBP, DBP, ALP, ALT and urinary proteins were significantly elevated while blood Hb was significantly lower in PE patients when compared to controls. In addition, identification of *FokI* rs2228570 polymorphism of *VDR* gene in PE patients suggests the involvement of *VDR* gene in PE pathology. Current study shows the importance *FokI* polymorphism of *VDR* gene and also reveals that this polymorphism may be associated with PE risk. Our results demands the molecular study of other important polymorphism including *FokI*, *ApaI* and *BsmI* of *VDR* gene as well as other PE susceptibility genes to check their role in PE pathology that will pave the way for in time patient diagnosis and management to avoid adverse outcomes.

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Annexure I

Turnitin Originality Report

Analysis of non-genetic risk factors and FokI polymorphism of Vitamin D Receptor Gene in etiology of preeclampsia cases presented at CMH, Muzaffarabad by Iram Shehzadi .

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