# INSULIN RESISTANCE IN FIRST TRIMESTER AND SUBSEQUENT DEVELOPMENT OF PREGNANCY INDUCED HYPERTENSION



**PhD Thesis**

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Reg no: 34/2004-2005



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**2008**

***Dedicated***

***to***

***My Beloved Parents***

# DECLARATION

The thesis entitled **'Insulin resistance in first trimester and subsequent development of pregnancy induced hypertension'** is submitted by **Dr Romena Helal** in partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD), under the Faculty of Postgraduate Medical Sciences and Research, University of Dhaka, Bangladesh. The study has been carried out in Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Dhaka during the period of January 2005 to January, 2008.

Part of this work has no been submitted for any degree or qualification in any other Institutes.

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# SUMMARY

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Pregnancy induced hypertension (PIH), consisting of preeclampsia or PE (proteinuric hypertension) and gestational hypertension or GH (non-proteinuric hypertension) are among the major causes of mortality and morbidity both in the mother and fetus. It affects 5-10% of pregnancies worldwide with higher frequencies in developing countries. Both maternal and fetal factors, from genetic and life style origins, are now implicated in the pathogenesis of PIH, but the precise causal agents and the natural history of the disorder are yet to be clarified.

One of the major converging point in the understanding of the pathophysiology of PIH is the involvement of insulin resistance which, in turn, is associated (either as a cause or effect) with a number of other risk factors or possible causal agents like obesity, dyslipidemia, subclinical chronic inflammation and endothelial dysfunction. Association of PIH with various markers of these abnormalities are well established in cross-sectional studies. However, longitudinal or prospective studies to explore the more causal relationship as well as predictive roles of these factors are still not decisive. In particular, studies relating these abnormalities to different indices of insulin resistance in a prospective design are relatively rare. The lesson from these studies also suggest a considerable racial heterogenicity possibly arising from both genetic and lifestyle (including environmental) factors. These facts necessitate such kind of studies among Bengali population who constitute the eighth largest population in the world speaking a single language (Bangla). Although the association of insulin resistance with already diagnosed PIH has been demonstrated in a cross-sectional study in this population, the prevalence and risk factors as well as causal relationship of the disorders have never been studied. Such data is invaluable from public health perspective to design cost-effective intervention for preventing the onset of PIH.

In the above perspective the present work was undertaken to investigate the prevalence as well as the anthropometric, clinical and biochemical risk factors of PIH in an urban hospital based Bengali population to address the possible causal role of insulin resistance and its covariates, and also to explore whether one or more index(ices) of insulin resistance or any of its covariates, measured in early stages of pregnancy, can predict the development of PIH at the later stages of gestation.

A nested case-control design was used in the study that allowed prevalence and risk factor analysis at the point of outcome (as in a cross-sectional design) and, at the same time, causal analysis as well as calculation of predictive values as in a prospective study. Initially, 430 pregnant subjects at their 1$^{st}$ trimester of pregnancy were recruited from various public hospitals of Dhaka city. The exclusion criteria included pregnancy with diabetes mellitus, chronic hypertension, chronic renal disease, multiple pregnancies, use of antifolate drugs (antiepileptics), smoking, alcohol intake and major medical problem. Among the recruited women 26 could not be followed up until the end of the pregnancy and were excluded from analysis. Thus a total of 404 pregnant subjects were included in the study and they were followed up for the development of PIH until delivery. PIH (and subgroups PE and GH) was diagnosed by standard criteria and proteinuria was diagnosed by protein-creatinine ratio. By matching the age- and gestational weeks with PIH cases a control group was defined out of the Non-PIH subjects. Serum insulin level was measured by microparticle enzyme immunoassay (EIA), serum SHBG (Sex Hormone Binding Globulin) by chemiluminescent immunometric assay and Insulin was measured by ELISA technique Insulin secretory capacity (HOMA%B) and insulin sensitivity (HOMA%S) were assessed by Homeostasis Model Assessment (HOMA). Urine total protein was measured by pyrogallol red method and urine creatinine was estimated by alkaline-picrate methods. The urine protein-creatinine ratio was obtained by dividing the urine protein concentration (mg/l) by the urine

creatinine concentration (mg/dl). Group analysis was made in terms of PIH and non-PIH cases as well as PIH and control cases. Association between variables were analyzed by bivariate as well as multivariate analysis. McNemar test was done to calculate sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the target parameters. PE and GH cases were compared to see possible difference between the subgroups. Finally, association of the predictive marker with other variables in early pregnancy were analyzed.

The frequency of PIH was found to be 10.14% in the present population. Analysis of the subgroups showed a prevalence of 7.67% in GH and 2.47% in PE groups. In group comparison analysis of PIH and non–PIH cases among all the recruited patients, PIH was found to occur in significantly higher frequency in multipara subjects (15.9% in multipara vs 7.4% in nullipara, p=0.027). The association of parity was sustained in multivariate (logistic regression) analysis. No other parameter was found to be associated with PIH when logistic regression was applied to all the 404 subjects.

When the PIH cases were matched for age and parity 101 non-PIH subjects could be recruited as Control (following a nested case-control design). On case-control comparison most of the indices of early pregnancy insulin sensitivity were found to be significantly lower in the PIH group compared to the Control [Glucose-Insulin ratio, median (range), 0.69 (0.44-1.30) in PIH vs 0.80 (0.39-1.67) in Control, p=0.004; QUICKI, 1.90 (1.64-2.48) in PIH vs 2.01 (1.59-2.79) in Control, p=0.002; HOMA%S, 110 (51-243) in PIH vs 155.9 (56.3-418.6) in Control, p=<0.001; and SHBG (nmol/l, 170 (10-198) in PIH vs 180 (103-219) in Control, p=0.003]. Among the covariates of insulin resistance BMI [23.7±4.1 in PIH vs 22.4±3.4 in control, p=0.047] and UPr/Cr 9.6 (3.48-20.5) in PIH vs 4.39 (1.1-15.5) in control, p=<0.001]. Other covariates of (TG, Choi, HDL, LDL) of insulin resistance did not show any significant difference between the two groups.

Correlation analysis between 3rd trimester blood pressure and 1st trimester SHBG as well as UPr/Cr indicated that PIH is associated with early pregnancy insulin resistance and endothelial damage. SHBG and UPr/Cr were found to be strongly correlated to each other.

Logistic regression analysis provided more confirmatory evidence on the association of PIH with SHBG (p=<0.001) and UPr/Cr (p=0.013) after adjusting the effect of the confounders.

The value of the early pregnancy levels of these markers for predicting the development of PIH in the late pregnancy were explored. At an optimum cut-off value of 180 nmol/l the sensitivity, specificity, PPV and NPV of SHBG were found to be 63%, 80%, 39% and 80% respectively. At an optimum cut-off value of 6.79 the corresponding values for UPr/Cr were 65%, 78%, 55% and 84%. PE and GH differed in the sensitivity and specificity of prediction by SHBG and UPr/Cr with higher predictivity of the insulin sensitivity index in PE and lower predictivity of the endothelial damage marker in GH.

It may be concluded that urban hospital based Bangladeshi pregnant population have 10.14% prevalence of PIH. Multiparity and overweight increase the risk of PIH, and insulin resistance and endothelial damage seem to be causally associated this disorder. The data also suggest that early pregnancy SHBG and UPr/Cr can be clinically useful in predicting future development of PIH in late stages gestation. Among these two options, UPr/Cr has a little better sensitivity and specificity and this can be preferred as it is technically much simpler (implementable even in rural settings) and cost-wise more economic.

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| | |
|---|---|
| ACOG | American College of Obstetrics & Gynecology |
| ANC | Antenatal Check-up |
| ADP | Adenosine di phosphate |
| ATP | Adenosine Tri phosphate |
| BIRDEM | Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders |
| BIRPERT | Bangladesh Institute of Research for Promotion of Essential and Reproductive Health and Technologies. |
| BMI | Body Mass Index |
| BP | Blood Pressure |
| BSMMU | Bangabandhu Sheikh Mujib Medical University |
| Chol | Total Cholesterol |
| CP | C-Peptide |
| CRP | C-Reactive Protein |
| DBP | Diastolic Blood Pressure |
| dl | Deciliter |
| DM | Diabetes Mellitus |
| $E_2$ | Estradiol |

| | |
|---|---|
| EOC | Emergency Obstetric Care |
| FFA | Free Fatty Acid |
| GH | Gestational Hypertension |
| GIR | Glucose Insulin Ratio |
| HDL | High Density Lipoprotein |
| HOMA B | Homeostasis Model Assessment β-Cell Function |
| HOMA S | Homeostasis Model Assessment Insulin Sensitivity |
| IL-6 | Inter Leukin-6 |
| IUGR | Intrauterine Growth Restriction |
| Kg | Kilogram |
| L | Liter |
| LDL | Low Density Lipoprotein |
| M | Meter |
| MEIA | Microparticle Enzyme Linked Immunoassay |
| mL | Milliliter |
| MBP | Mean Blood Pressure |
| nmol | Nenomole |
| OPD | Out Patient Department |
| PAI-I | Plasminogen Activator Inhibitor-I |
| PAPP-A | Pregnancy Associated Plasma Protein-A |
| PE | Preeclampsia |
| PIH | Pregnancy Induced Hypertension |

| | |
|---|---|
| PRL | Prolactine |
| QUICKI | Quantitive Insulin Sensitive Check Index |
| RV | Reaction Vessel |
| ROC | Receiver Operator characteristic Curve |
| SBP | Systolic Blood Pressure |
| SD | Standard Deviation |
| SHBG | Sex Hormone Binding Globulin |
| SPSS | Statistical Packaged for Social Sciences |
| T | Testosterone |
| TEAH | Tetraethyl ammonium hydroxide |
| TG | Triglyceride |
| TNFα | Tumor Necrosis Factor Alfa |
| TPA Ag | Tissue type Plasminogen Activator Antigen |
| µl | Microliter |
| µmol | Micromole |
| UPr/Cr | Urine Protein Creatinine Ratio |
| VCAM-1 | Vascular Cell Adhesion Molecule -1 |

# Chapter 1

# INTRODUCTION

# 1. INTRODUCTION

## a. Hypertension in pregnancy

Hypertension, a common disorder in pregnancy, is the leading cause of maternal, fetal and neonatal morbidity and mortality all over the world (Allen et al, 2004; Preeclampsia Foundation, 2000). Pregnancy may induce hypertension in women who are normotensive before pregnancy and it may aggravate hypertension in those who are hypertensive before pregnancy. Diagnosing and managing hypertension in pregnancy consume a significant amount of resources and can challenge even the most experienced clinician (Emery, 2005). Pregnancies complicated by hypertension are associated with increased risk of adverse fetal, neonatal and maternal outcomes, including preterm birth, intrauterine growth restriction, perinatal death, acute renal or hepatic failure, antepartum hemorrhage, postpartum hemorrhage and maternal death (Report of the National High Blood Pressure Education Program Working Group, 2000; Brown et al, 2000, Collins & Wallenburg, 1989). The combination of hypertension plus proteinuria markedly increases the risk of perinatal morbidity and mortality over hypertension alone (Friedmen, Taylor & Roberts, 1976).

The worldwide incidence of the disorder is still high in spite of the significant improvement of mother and childcare over the last decades. Hypertensive disorders complicate 12% to 22% of all pregnancies, with results that range from inconsequential to catastrophic (Emery, 2005). They are responsible for 17.6% of

maternal deaths, making them the third leading cause after thromboembolism and hemorrhage (Emery, 2005).

Hypertension in pregnancy includes the following disorders as per definitions from Solomon & Seely (2001):

*Pregnancy Induced Hypertension (PIH):* Gestational Hypertension (GH) and Preeclampsia (PE) are together considered as pregnancy induced hypertension or PIH

- *GH (GH):* Blood pressure elevation detected for the first time after midpregnancy and distinguished from PE by the absence of proteinuria (non-proteinuric hypertesion).
- *Preeclampsia (PE):* Hypertension developing after 20 weeks of gestation accompanied by significant proteinuria (protenuric hypertension).

*Chronic Hypertension:* Elevated blood pressure in the mother predating pregnancies. It can also be diagnosed in retrospect when PE or GH fails to normalize after pregnancy is over.

*Eclampsia:* Occurrence, in a woman with PE, of seizures that cannot be attributed to other causes.

**b. Pregnancy induced hypertension**

PE and GH are together considered as pregnancy induced hypertension or PIH (Solomon & Seely, 2001). According to the National High Blood Pressure

Education Program (2000) PE and GH may represent different manifestations of one disease process, although there is some evidence that these conditions may be pathophysiologically distinct (Fisher et al, 1981). Whether GH and PE represent different ends of a single pathophysiological spectrum or two distinct processes linked coincidentally with hypertension is still unknown (Myles et al, 2002). In the absence of severe disease manifestations, discrimination between PE and GH may be difficult. This distinction is often made solely on the basis of urine protein determination, frequently by dipstick protein measurement, which is recognized to be an imperfect surrogate for 24-hour measurements (Meyer et al, 1994). For research purposes especially, a rigorous classification scheme is advocated in which PE is distinguished from transient gestational hypertension by lack of significant proteinuria in the latter disorder (Lindheimer et al, 1999).

Although PE is widely recognized as a leading cause of maternal and fetal morbidity and mortality, GH is often considered a benign condition. However, although maternal end-organ damage is more common in PE, GH is also associated with increased rates of cesarean section, preterm delivery, and small-for-gestational age babies (Thadhani et al, 2001; Gofton et al, 2001; Buchbinder et al, 2002). The frequency of poor maternal and infant outcomes associated with PE may explain the high rate of elective delivery in women with this condition. The small-for-gestational age rate associated with GH was lower than that associated with PE, suggesting that *in utero* growth is more affected when there is proteinuric hypertension (Roberts et al, 2005). Clinicians need to be aware that

proteinuria associated with chronic hypertension identifies a pregnancy at increased risk. In such cases, closer maternal and fetal surveillance is indicated, together with referral to a higher level of care if necessary (Lindheimer et al, 1985).

While maternal diastolic blood pressure (DBP) greater than 110 mm Hg is associated with an increased risk for placental abruption and fetal growth restriction, superimposed preeclamptic disorders cause most of the morbidity due to chronic hypertension during pregnancy. Severe maternal complications include eclamptic seizures, intracerebral hemorrhage, pulmonary edema due to capillary leak or myocardial dysfunction, acute renal failure due to vasospasm, proteinuria greater than 4-5 g/d, hepatic swelling with or without liver dysfunction, and disseminated intravascular coagulation and/or consumptive coagulopathy. Fetal complications include abruptio placentae, intrauterine growth restriction, premature delivery and intrauterine fetal death (Gibson, Carson & Michael, 2007). PE presents a potential danger to the mother and infant, and, where time allows, a high level of maternal and neonatal medical care should be sought (Report of the National High Blood Pressure Education Program, 2000).

The frequency of the disorders is substantially affected by parity. PIH occurs in about 16-24% of first pregnancies and 12-15% of subsequent pregnancies (Robson, 1999). PE complicates 3-5% first pregnancies and 1% of subsequent pregnancies with around 5-10% cases being severe (Robson, 1999). Using population-based data (Gibson, Carson & Michael, 2007) reported that

approximately 1% of pregnancies are complicated by chronic hypertension, 5-6% by GH (without proteinuria), and 1-2% by PE (Gibson, Carson & Michael, 2007).

PE is responsible for approximately 50,000 maternal deaths yearly worldwide, 25% of all cases of fetal growth restriction, and 15% of preterm births in developed countries (Roberts, 1998; Duley, 1992; Goldenberg & Rouse, 1998). PIH is a highly important public health problem in developed countries. It is the main cause of maternal mortality in these countries and is associated with a 5-fold increase in perinatal mortality (Lopez- Jaramilleo, Casas & Serrano, 2001). Mortality from hypertensive disorders is much higher reaching rates of 70-120 per 100 thousand maternities (Robson, 1999). A large portion of the perinatal mortality is consequently due to iatrogenic prematurety, up to 15% of preterm births are results of PE (Meis, Goldenberg & Mercer, 1998).

The incidence of PE in developing countries is particularly high due to lack of proper care of the mother during pregnancy. Geographic, social, economic and racial differences are responsible for an incidence that is up to three times higher in some populations. In developing countries, PE effects 4.4% of all deliveries and may be as high as 18% in some settings in Africa (Moodley, Mphatsoe & Gouws, 1999).

The incidence of eclampsia is extraordinarily high in Bangladesh-7.9% (not including PE), according to the results of a house-to-house survey (BIRPERT, 1994). In this country, only 2.3% women end their pregnancy under medical supervision (whether it is abortion or delivery) (Yasmin, Rahman & Chowdhiry,

1995), the rest have no access to obstetric care. As a result, most PE cases remains unrecognized until severe complications, such as eclampsia, occur. In a baseline survey for assessment of emergency obstetric care (EOC) in Bangladesh, 5% of total obstetrical admissions in health facilities were due to PE and eclampsia (Yasmin, Rahman & Chowdhiry, 1995). As there are approximately 3.6 million births per year in Bangladesh, over 100,000 women develop eclampsia per year. Eclampsia contributes 16% of maternal mortality on a national basis, which is equivalent to about 4500 maternal deaths in one year (Fauveau et al, 1988). There is, however, no large scale population based epidemiological data on the incidence or prevalence of PE and GH in Bangladesh.

## c. Etiopathogenesis of PIH

### i. Risk Factors

Despite the significant morbidity associated with hypertensive disorders in pregnancy, the pathogenesis remains unclear, which limits the ability to prevent and treat this disorder. The cause of PE is unknown, although several factors have been shown to contribute. PE is more common in women during their first pregnancy (Mounier-Vehier, Equine & Valat-Rigot, 1999) as well as in women who are obese (Mounier-Vehier, Equine & Valat-Rigot, 1999; Sibai et al, 1997), who have diabetes or who have GH (Persson & Hanson, 1998; Saudan et al, 1998). Women who have had PE during a previous pregnancy are also at increased risk (Myatt & Miodovnik, 1999).

Some researchers suggested that PIH is more likely to occur at both extremes of reproductive age (over 40 or under 18 years of age), but is greatest in women younger than 20 years of age (Saftas et al,1990; Chesley, 1984). In a review by Saftas et al (1990), white women and African women, 15 to 17 years of age, were 2.6 times and 2.4 times more likely, respectively, to develop PE than their 25-to 34-year old counterparts.

Additional risk factors for PIH have been reported. Women with gestational diabetes have 15% with pregestational diabetes have a 30% risk of PE women with renal disease, greater than 30% Body Mass Index (BMI), polycystic ovarian syndrome, lupus or other autoimmune disorders such as rheumatoid arthritis, sarcoidosis or multiple sclerosis, also have increased risk of PE of varying differ (Preeclampsia Foundation, 2000).

Other factors, such as interval between births (Skjaerven et al, 2002), change of partner between births (Skjaerven, Wilcox & Lie, 2002; Dekker, Robillard & Hulsey, 1998), chronic hypertension (Sibai et al, 1998) and smoking in pregnancy (Cnattingius et al, 1997) have also been associated with the risk of PE.

Women with PE and hyperuricemia have a more severe form of PE with an increase in preterm births and smaller infants for gestational age (Redman et al, 1976; Fadel, Nothrop & Misenhimer, 1976; D'Anna et al, 2000; Wakwe & Audu, 1999). Women who were born after a pregnancy affected by PE are themselves at increased risk of PE in their own pregnancies (Chesley & Cooper, 1986; Cooper et al, 1988; Arngrimsson et al, 1990). Placental dysfunction could cause PE in babies

with low weight for gestational age (Ness & Roberts, 1996; Teasdale, 1985; Ghidini et al, 1997), but is less likely for larger babies (Odegard et al, 2000; Xiong, 2002).

### ii. Pathophysiology

Although PIH was first described over 100 years ago, little is known about the pathophysiology of this disease. It is now well acknowledged that the cause(s) of PIH include immune, genetic, nutritional and placental abnormalities (Seely & Solomon, 2003). Knowledge in this regard is mostly concentrated on PE, but very often GH was not clearly separated from PE in these studies and the evidences mostly apply for PIH as a whole. Chesly in 1978 described PE as a 'disease of theories' because of its obscure causes. Several theories, which are not mutually exclusive, attempt to explain the pathophysiology of PE.

#### Immunologic factors

The increased incidence of PE observed in patients using barrier contraception, in multiparous women conceiving with a new partner, and in nulliparous women suggests an immunologic role (Matthew, 2003). Several investigators have shown that the incidence of PE in multiparous women is lower than primiparous women, but higher if the multiparous woman has a different partner (Li & Wi, 2000; Trupin, Simon & Esknazi, 1996). Repeated exposure to sperm from the same individual may also be a preventive factor in the development of PE (Skjaerven, Wilcox & Lie, 2002).

## Genetic factors

Inheritance pattern analysis supports the hypothesis of transmission of PE from mother to fetus by a recessive gene (Matthew, 2003). A large number of studies suggest a genetic susceptibility to PE by showing that daughters of women with PE are 4-5 times more likely to develop the syndrome than daughters-in-laws (Robson, 1999). There is considerable evidence of genetic inheritance of severe PE which may be simple recessive trait involving fetal and maternal genotype, multifactorial inheritance, incomplete penetrance of the gene or variable susceptibility to fetal challenge (Robson, 1999).

It is most unlikely that there is a single PE gene. There is a cluster of polymorphisms which, possibly in conjunction with environmental factors, predispose to the development of the condition (Brougton, 1999). Accurate phenotyping is vital for any genetic studies of PE but it is particularly difficult since the disease is only clinically detectable in the second half of pregnancy. It is increasingly likely that there is a fetal genetic contribution which can only be examined after birth. Candidate genes are examined on the basis of displayed or hypothetical pathophysiological effects, but so far no confirmed evidence of association or linkage has been found. The probable genes include HLA-DR beta, HLA-G and tumour necrosis factor alpha (chromosome 6), angiotensin- converting enzyme (chromosome 17) and Cu-Zn superoxide dismutase (chromosome 21). Chromosomal exclusion mapping and pedegree studies suggest a role for the genes on chromosones 1,3,4,9 or 18. Two genes concerned with clotting, those

for factor 5 and methylenetetrahydrofolate reductase, lie on chromosome 1. Both have polymophisms present in significantly higher frequency in women with PE and they also show functional abnormality. They probably predispose to the development of the condition without being necessary for it. The angiotensinogen (Aogen) gene also lies on chromosome 1. The renin-angiotensin system may be activated during the early stages of PE and subsequently suppressed. In some populations, a relatively common polymorphism is present in raised frequency in women with PE, but it is also raised in non-pregnant hypertensive subjects. However, it is in partial linkage disequilibrium with another polymorphism, which shows significantly distorted transmission from mother to fetus in PE pregnancies. Furthermore, its expression is significantly raised in the decidual spiral arteries; abnormal placentation is a feature of PE. It has also been shown that a relatively common polymorphism in the angiotensin AT1 receptor gene (chromosone 3) is associated with raised density of the receptor (Broughton, 1999).

A familial tendency to PE is well recognized due to inherited variation of angiotensinogen gene (Robson, 1999). These findings on racial variation of the disease may indicate substantial genetic heterogenicity for the disease. The association with a common molecular variant of the AGT (Angiotensinogen gene), in which methionine is substituted for threonine (T2 35) at residue 235, has been reported in both Caucasians and Japanese (Kobashi et al, 1999). The region of the chromosome 2P 25 and 9P 13 may harbor susceptibility genes for PE and the

locus at 9P 13 has been shown to be a candidate region for type 2 diabetes in Finland and China (Laivuori, Tikkanen & Ylikorkala, 2003).

## Nutritional factors

PIH has been proposed to occur secondary to malnutrition (Dudek, 2001). However, Clausen et al (2001) reported that energy intake was higher in women with PE. Despite the belief that low protein intake is associated with an increased risk of PE, a number of studies showed that reduced protein intake is not related to PE and protein supplementation did not reduce the incidence of PE (Belizan et al, 1983). Intake of calcium (Belizan & Villar,1980; Ramos et al, 2006; Villar et al, 2003), zinc (Villar et al, 2003), folate (Belizan et al, 1983; Scholl & Johnson, 2000) and vitamin C and E (Belizan et al, 1983; Banerjee, Chambers & Campbell, 2006; Rumiris et al, 2006) have been suggested to be associated with preeclampsia. Wacker et al (2000) showed a high prevalence of preeclampsia in a group of pregnant women with riboflavin deficiency. This deficiency was positively correlated with the development of preeclampsia even when controlled for parity, maternal age, weight and gestational age. Riboflavin deficiency was more common towards the end of pregnancy. Riboflavin status often decreases at the end of pregnancy because placenta formation depends on a reproduction-specific riboflavin carrier protein (Wacker et al, 2000).

## Placental factors

There are some data suggesting that primary cause of PE is placental abnormalities. Association of PE with abnormalities in placental perfusion is a well-accepted phenomenon. A recent prospective study has shown that abnormalities in placental blood flow in early pregnancy (as evaluated by color Doppler) are associated with increased incidence of PE in late pregnancy. The major cause of fetal compromise is reduced uteroplacental perfusion (Hauth & Cunninghum, 1999). The only intervention that effectively reverses the syndrome is delivery. The syndrome is polymorphic in that virtually every organ system can be affected. It can cause blood pressure to rise and can put the patient at risk of stroke and impaired kidney function, impaired liver function, blood clotting problem, pulmonary edema (fluid lungs), seizures and, in severe forms, maternal and infant death. Because PE affects the blood flow and placenta, babies can be smaller and are often born prematurely. Abnormal placentation is clearly involved in the genesis of both preeclampsia and fetal intra-uterine growth restriction (Redman, 1991). PE is a systemic disorder that occurs in presence of placenta. Since with delivery of placenta the problem disappears the placenta is thought to be the key to its pathogenesis (Brosens, 1977). During normal pregnancy, the fetal allograft interacts with maternal decidua and an apparent state of mutual immunological tolerance develops. Cytotrophoblast invade the uterine spiral arteries reaching up to decidual segment by 4-6 weeks and distal third of myometrium by 16-18 weeks. The endovascular trophoblastic cells invade the maternal spiral arteries,

where they replace the endothelium and destroy the medial elastic and muscular tissue of arterial wall. The end result is that thin walled muscular spiral arteries are converted into sac like flaccid uteroplacental vessels, which passively dilate to accommodate the greatly augmented blood flow required in pregnancy.

PE develops following a partial failure in the process of placentation. In these cases the vessels fail to dilate and remain unresponsive to vasomotor influences leading to high resistance that lowers the circulatory flow, which is the earliest and most consistent change in PE (Shankin & Sibai, 1989).

**Endothelial Dysfunction**

Maternal endothelial cell dysfunction is the event resulting in the diverse clinical manifestation of PE (Roberts et al, 1990). Evidence has show accumulated to support a major role of the endothelium in PE (Taylor & Roberts, 1999). The mechanisms involved in induction of endothelial cell dysfunction are poorly understood in PE (with or without IUGR); however, is distinguished from IUGR (without PE) by extension of disturbance into the maternal vasculature (Friedmen, Taylor & Roberts, 1991). It has been proposed that product(s) of fetal-placental unit enter the circulation and then initiate the maternal pathophysiologic changes of PE (Roberts et al, 1990). However there is evidence that both feto-placental and maternal factor interact in manifesting endothelial cell dysfunction and its clinical manifestations (Ness & Roberts, 1996).

There are accumulating evidences that deficient trophoblast invasion of placental bed leads to poorly perfused fetoplacental unit (Brosens, 1977). This results in secretion of factors into maternal circulation which causes activation of vascular endothelium, with the clinical syndrome resulting from widespread changes in endothelial cell functions (Robert, 1998). These may lead to vascular injury and vascular pathology with changes in vasomotor tone and coagulation. The vascular endothelial dysfunction results in increased permeability, hypercoagulability, and diffuse vasospasm. Therefore endothelial activation in PE includes characteristics changes in glomerular capillary endothelial morphology, elevated blood level molecules associated with endothelial cell activation like endothelin and cellular fibronectin (McCartney, 1969; Brown, Zammit & Lowe, 1989; Taylor et al, 1991). The biochemical markers of endothelial dysfunction, including E-selectin, intercellular cell adhesion molecule-1, von Willebrand factor, and thrombomodulin and PAPP-A (Elhadd et al, 2001).

It has been postulated that carbohydrate and lipid abnormalities could play a role in the pathogenesis of PE causing altered endothelial function and vascular damage (Sower, Saleh & Sokol, 1995; Sattar, Gaw & Packard, 1996) and it is well known that vascular dysfunction is associated with hypertension in pregnancy (Rodgers, Taylor & Roberts, 1988; Pinto & Sorrentino, 1991; Roberts & Redman, 1993).

In the recent years some interest has been observed regarding the urinary protein/creatinine ratio, a marker for microvascular damage, for early detection of

PE. Studies have suggested an excellent correlation between the random urinary protein/creatinine ratio and 24-hours urinary total protein level (Bolero, Bella & Bleacher, 1987; Robert et al, 1997). Few studies have evaluated the usefulness of protein/creatinine ratio as a screening tool for the evaluation of proteinurea in women with suspected PE (Rodriguez-Thompson & Lieberman, 2001; Jaschevatzky et al, 1990; Young, Buchanan & Kinch, 1996). Although several studies have shown a strong linear association between the random urinary protein-to-creatinine ratio and 24-hours total protein excretion in pregnant women (Villar & Sabai, 1989; Bolero, Bella & Bleacher, 1987; Jaschevatzky et al, 1990; Kalpan, Jack & Opheim, 1989) till now a few studies were done to evaluate whether urinary protein-creatinine ratio in first trimester can be used as an early marker for PIH (Ragip et al, 2004).

## Circulating factors

There is substantial evidence to suggest that there is a factor in the serum of women with PE which perturbs endothelial function. Circulating factors are Lipid peroxidation degradation product, reactive oxygen species and cytokines (IL-6) are known to cause endothelial dysfunction (Robson, 1999).

## Hyperdynamic circulation

The vascular, cardiovascular and associated hemodynamics play an important role in PE. Vascular constriction with segmental spasm that occurs particularly in arterioles leads to higher resistance to blood flow and higher arterial pressure reported by Gabrriella and Jules (2002). It has been postulated that a high-output, low resistance phase seen in the preclinical period of the disorder transform into a low-output, high resistance at the time of diagnosis. Another model suggests that the increase in maternal cardiac output, rather than increase peripheral vascular resistance, is the commonest hemodynamic feature in PE (Easterling, Benedetti & Schmucker, 1990).

## Renal lesion

The most characteristic morphological abnormality in PE is a renal lesion termed as glomerular endotheliosis where there is swelling of glomerular capillary endothelial cells. This is present in more than 70% of primipara women with PE. It reverses completely after delivery (Spargo, McGartney & Winemiller, 1959). The prominent clinical feature of PE is edema and glomerular capillary protein leakage with consistent loss of normal endothelial transport. The parameter of vascular endothelial injury are fibronectin, NO, plasminogen activator and $\beta_2$ microglobulin.

## Oxidative stress

One hypothesis receiving increased attention is that placental and maternal free radical reactions promote a cycle of events that compromise the defensive functioning of the vascular endothelium in PE which is commonly known as oxidative stress hypothesis (Hubei et al, 1989). Oxidative stress may cause widespread endothelial cell dysfunction, which can form the common link. There is abundant morphological, functional, and biochemical evidence to support this hypothesis. It has also been hypothesized that reduction in the antioxidant activity may enhance endothelial cell oxidative damage (lioka, 1994; Mikhail et al, 1994; Kwasniewska, Tukendorf & Semczuk, 1998).

## Lipids and Free fatty acid

In women with established PE, triglyceride (Belo et al, 2002; Bartha et al, 2002; Kaaja et al 1999) and free fatty acid levels (Kaaja et al, 1999; Hubei et al, 1996) have been reported to be higher and high-density lipoprotein cholesterol levels lower (Belo et al, 2002; Kaaja et al, 1999) than those in women with normotensive pregnancy. Other investigators have documented these abnormalities only in women with GH (Caruso et al, 1999). Studies have reported an increased proportion of small dense LDL particles in women with established PE (Belo et el, 2002; Ogura et al, 2002). Oxidized lipids may impair endothelial function directly or indirectly by effects on prostaglandins, including increasing synthesis of thromboxane and inhibiting synthesis of prostacyclin (Bruckdorfer,

1996). Increases in small dense LDL and triglycerides may also contribute to impaired endothelial function.

High-density cholesterol is the antiatherogenic lipoprotein and appears to modulate endothelial function in a beneficial fashion (Gotto, 2001). It is well recognized that low HDL is associated with increased cardiovascular risk (Gotto, 2001). Increased FFA levels are known to negatively affect endothelial function (Steinberg et al, 1997).

**Inflammatory markers**

An increased level of C-reactive protein (CRP) is a sensitive marker of systemic inflammation that is associated with cardiovascular disease (Ridker et al, 2000) and PE (Wolf et al, 2001). In another study CRP was not predictive of PE (Djurovic et al, 2002). Plasma levels of interleukin-6 (IL-6) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) have been shown to be raised in PE (Greer et al, 1994; Conrad, Miles & Benyo, 1998; Vince et al, 1995). It has been suggested that PE is attributable to an excessive maternal inflammatory response to pregnancy secondary to a combination of placental factors and maternal factors related to phenotype and genotype (Redman, Sacks & Sargent, 1999). This inflammatory response contributes to the wider syndrome of endothelial dysfunction and thrombotic and metabolic disturbances seen in PE (Dilys et al, 2004).

## Pregnancy associated proteins

The concentration of several placental proteins, inflammatory cytokines and growth factors are altered in the maternal circulation of women with PE. Inhibin (α-β) dimmer and activin (β-β) are glycoprotein hormones belonging to the transforming growth factors β family. Follestatin is a high affinity activin binding protein. Several reports have previously shown that the level of activin A (βA- βA) dimmer and inhibin A (α-βA) dimmer are significantly elevated in circulation of women who have developed PE (Muttukrishna et al, 1997; Petraglia et al, 1995; Fraser, McAsey & Coney, 1998) and in women who subsequently develop PE compared with gestational age-matched control pregnant women (Cuckle, Sehmi & Jones, 1998; Silver et al, 1999). The possible occurrence of abnormal serum level of the new generation pregnancy protein, the pregnancy associated plasma protein A (PAPP-A), has previously been studied in details and raised PAPP-A concentration in eclampsia have been reported earlier (Toop & Klopper, 1981; Hughes et al, 1980). In the recent years there are some works on PAPP-A to investigate whether it can be associated with PE. There are still some controversies on this issue. Some investigators reported that preeclampsia was associated with increased maternal serum level of PAPP-A (Bersinger et al, 2003; Toop & Klopper, 1981), while others found similar level of PAPP-A in preeclampsia group compared to that of controls (Barnea et al, 1986).

## Insulin resistance and associated biomarkers

Insulin resistance is associated with hyperglycemia, hyperinsulinemia, and dyslipidemia (Reaven, 1996). More recently, it has been recognized that the insulin resistance syndrome may also be involved in other metabolic abnormalities, including increased concentrations of plasminogen activator inhibitor (PAI-1) (Abbasi et al, 1999); leptin (Segal et al, 1996) and tumor necrosis factor-a (TNF-α) (Fernandez et al, 1998). Although these markers are surrogate measures of insulin sensitivity, observed associations between many of these markers and PIH risk further suggest a role for insulin resistance in the development of PIH.

## Tumor Necrosis Factor-α

Elevations in TNF-α (Yoneyama et al, 2002; Vince et al, 1995; Visser et al, 2002) or its receptor (Visser et al, 2002) have been reported in women with established PE compared with normotensive controls. TNF-α may promote hypercoagulability and increased lipolysis, with resulting impairment of endothelial relaxation. TNF-α also causes the release of VCAM-1, elevations of which have been reported in established PE (Visser et al, 2002; Phocas et al, 2000), although not before its development (Clausen et al, 2000).

## Plasminogen Activator Inhibitor-1 and TPA Ag

Women with established PE have higher levels of tissue plasminogen activator antigen (TPA Ag) than normotensive pregnant women, and elevations are proportional to the magnitude of proteinuria (Bel et al, 2002). PAI-1 is likewise elevated in established PE and is higher in more severe disease (Shaarawy & Didy, 1996). Among women at high risk for PE, the ratio of PAI-1 to PAI-2 (the latter primarily produced by the placenta) was increased before the development of disease (Chappell et al, 2002). Increased PAI-1 may reflect impaired fibrinolytic function, which might predispose to the coagulopathy associated with PE.

## Leptin

Some data suggest that leptin levels are elevated in women with established preeclampsia (Anim-Nyame et al, 2000; McCarthy, Misra & Roberts, 1999; Vitoratos et el, 2001), although not in women with GH (Vitoratos et al, 2001). Leptin levels as early as 20 wk gestation were reported to predict the development of PE in a high risk population (Chappell et al, 2002). Increased leptin levels may in part reflect maternal adiposity and have also been hypothesized to reflect placental insufficiency. Leptin might also contribute to endothelial dysfunction by increasing free fatty acid oxidation (Yamagishi et al, 2001).

### Testosterone and SHBG

Some cross-sectional data indicate higher levels of total and free testosterone, but comparable levels of SHBG, in women with established PE (Acromite et al, 1999). In a prospective study, neither total nor free testosterone in the first trimester predicted later development of PE (Wolf et al, 2002).

## d. Association of Insulin resistance

### i. Insulin resistance in normal pregnancy

Normal pregnancy is associated with hyperinsulinemia and insulin resistance. Insulin sensitivity decreases during pregnancy with peaks in the third trimester (Kuhl, 1991; Cousins et al, 1980; Catalano et al, 1993; Buchanan et al, 1990) and after delivery it returns to prepregnancy level rapidly (Yen, 1973). The basis of insulin resistance in normal pregnancy is not well understood. It is postulated that various hormonal changes in pregnancy, particularly human placental lactogen, cortisol, progesterone and estrogen are responsible for the loss of insulin sensitivity in this condition (Barbieri, 1999).

### ii. Insulin resistance in PIH

A major converging point in the pathogenesis of the disease is thought to be the deficiency of the action of insulin, the central hormone in the metabolic process. It

has been claimed that impaired insulin sensitivity is the underlying factor in developing the relative deficiency of insulin (Caruso et al, 1999).

Substantial evidence has now been accumulated to suggest that PIH is associated with greater degree of insulin resistance than characteristics of normal pregnancy. Evidence shows that individuals with PE may have clinically silent but persistent alteration in insulin resistance (Berkowitz, 1998). Recently interesting analogies between insulin resistance syndrome and the hypertensive state in pregnancy have been found. Some authors have suggested that hypertension in pregnancy is characterized by hyperinsulinemia (Bauman, Maimen & Langer, 1988; Sowers et al, 1995; Abundis et al, 1996), although a few studies claim that only GH and not PE is associated with insulin resistance (Caruso et al, 1999; Bartha et al, 1999), the overwhelming data supports the idea that both GH and PE are insulin resistant conditions (Seely & Solomon, 2003). The usual period of onset of PIH (late pregnancy) (Kuhl, 1991) corresponds with the maximal degree of insulin resistance and it supports a possible association. It has been suggested that insulin resistant subjects are at risk to develop cluster of abnormalities including high plasma concentrations of TG, a decrease in plasma high density lipoprotein (HDL) cholesterol concentrations and hypertension (Reaven & Banting, 1988).

The postulated mechanism by which hyperinsulinemia and insulin resistance could lead to hypertension are an associated increase in sympathetic nervous system activity or sensitivity to catecholamines, increased $Na^+$ reabsorption in the proximal renal tubule, altered cellular cation transport and associated endothelial

dysfunction (Rowe et al, 1981; Reaven, Lithell and Landsberg, 1996; Defronzo et al, 1975; Doria et al, 1991; Gibbons and Dzau, 1994).

Emerging evidence suggests that insulin resistance, which has been linked to essential hypertension, may play a role in PIH (Solomon & Seely, 2001). Insulin resistance syndrome provides a possible link between hypertensive pregnancy and many of its risk factors and sequels in both pregnancy and later life. The association of essential hypertension with insulin resistance and hyperinsulinemia has been well described (Reaven & Banting lecture, 1998; Ferrannini et al, 1987).

Conditions associated with increased insulin resistance, including gestational diabetes, polycystic ovary syndrome and obesity, may predispose to hypertensive pregnancy. Furthermore, metabolic abnormalities linked to the insulin resistance syndrome are also observed in women with PIH to a greater degree than in normotensive pregnant women. These include glucose intolerance, hyperinsulinemia, hyperlipidemia, and high levels of plasminogen activator inhibitor-1, leptin, and tumor necrosis factor-a (Caren et al, 2001). Increased incidence of cardiovascular diseases in diabetic patients is well known phenomenon, but sufficient data has now been accumulated to claim that insulin resistance is associated with a higher risk of cardiovascular problems even in the absence of diabetes. In women whose pregnancies are complicated by hypertension, there appears to be an exaggeration of insulin resistance and associated metabolic changes. Exaggerated hyperinsulinemia relative to normal pregnancy is well described in women with established preeclampsia (Kaaja et al,

1999; Lorentzen et al, 1998) or gestational hypertension (Kaaja et al, 1999). In a small study using the euglycemic clamp technique, insulin resistance was greatest in women with gestational hypertension, whereas results were similar in women with normotensive pregnancy and women with preeclampsia (Caruso et al, 1999). A recent study using minimal model analysis (Bartha et al, 2000) yielded comparable results, although in another report using this method, women with preeclampsia were more insulin resistant than normotensive controls (Kaaja et al, 1999). Several reports have documented hyperinsulinemia and/or hyperglycemia in early or midpregnancy, before the development of preeclampsia (Sermer et al, 1995; Joffe et al, 1998), gestational hypertension, or both (Solomon et al, 1994; Solomon et al, 1999; Sowers et al, 1996; Innes, Wimsatt & McDuffie, 2001).

Insulin resistance and/or associated hyperglycemia may impair endothelial function (Cales-Escandon & Cipolla, 2001). Obesity and physical inactivity, two factors closely associated with insulin resistance, are also predictive of hypertensive pregnancy. A higher body mass index before pregnancy or early in pregnancy is associated with increased risk for both preeclampsia and gestational hypertension (Solomon et al, 1994; Solomon et al, 1999; Thadhani et al, 1999; Sattar et al, 2001; Saftlas et al, 2000). Furthermore, greater gestational weight gain has also predicted risk for preeclampsia (Solomon et al, 1994) or gestational hypertension (Saftlas et al, 2000), as has higher waist circumference (a measure of central adiposity) between 6 and 16 wk (Sattar et al, 2001). In contrast, increased participation in leisure time physical activity in the first 20 wk of

pregnancy has been associated with reduced risk (Marcoux, Brisson & Fabia, 1989). Several features of the insulin resistance syndrome, such as obesity (Sibai, Ewell & Levine, 1997), hypertension (Thadhani et al, 2001), dyslipidemia (Thadhani et al, 1999), systemic inflammation (Wolf et al, 2001), and impaired fibrinolysis (Estelles et al, 1989), are also associated with preeclampsia.

It has been postulated that carbohydrate and lipid abnormalities could play a role in the pathogenesis of PE causing altered endothelial function and vascular damage (Sower et al, 1995; Sattar et al, 1996) and it is well known that vascular dysfunction is associated with hypertension in pregnancy (Rodgers, Taylor & Roberts, 1988; Pinto & Sorrentino , 1991; Roberts & Redman, 1993). There are, however, contradictory reports on the role of insulin resistance in PE showing that, as with other forms of secondary hypertension and, unlike essential hypertension, the pathophysiology of PE is not associated with insulin resistance (Roberts et al, 1998).

One of the major reasons for the above controversy may be the substantial genetic heterogenicity reported for PE (as mentioned earlier) as well as for insulin secretion and sensitivity (Alberti & Zinmmet, 1998). Deficiency of insulin action may very well arise from insulin secretory dysfunction, but no attention has so far given to the involvement of this possible mechanism in PE. For example, insulin resistance plays a more dominant role in developing DM in the Caucasian population where as insulin secretory defect seems to be a predominant factor in Bangladeshi population (Zinnat, 1997). Thus the issue of the factors underlying

deficient insulin action in PE is still an open question, particularly in our population.

Women with polycystic ovary syndrome or gestational diabetes, two disorders characterized by insulin resistance, are at increased risk of preeclampsia (De Vries, Dekker & Schoemker, 1998; Schmidt et al, 2001). Collectively, these data suggest that insulin resistance may contribute to the pathogenesis of preeclampsia (Solomon et al, 2001). Although insulin resistance is associated with preeclampsia the majority of evidence comes from cross-sectional and retrospective studies. For example, in studies that examined surrogate markers of insulin resistance, women with established preeclampsia displayed elevated levels of glucose (Innes, Wimsatt & McDuffie, 2001), uric acid (Kaaja et al, 1999), triglycerides (Wakatsuki et al, 2000), leptin (Teppa et al, 2000), plasminogen activating inhibitor-1 (Estelles et al, 1989) and reduced high density lipoprotein levels (Wakatsuki et al, 2000).

An increased risk of hypertension and stroke has been observed in women with history of gestational hypertension, similar to those observed in women with previous preeclampsia (Wilson et al, 2003). Reduced insulin sensitivity, altered angiogenesis and endothelial function, and a relative hyperandrogenemia found in women with previous PE have been indicated to contribute to their increased risk of cardiovascular diseases (Wolf et al, 2004; Chambers et al, 2001; Laivuori et al,1996). Women with hypertension in pregnancy had an augmented risk of death for cardiac ischemia in the later life than general population, with significantly

opposite to their diabetic counterparts regarding β cell secretory capacity, but they all showed a similar trend in case of insulin sensitivity. This fact further reinforces the previous conclusion that insulin resistance is a primary defect in our PIH population.

Several markers have been associated recently with the insulin resistance syndrome, including tumor necrosis factor (TNF)-α, IL, IL-6, platelet activator inhibitor (PAI-1), C-reactive protein (CRP), leptin, adiponectin and homocysteine (Palomo & Alarcon, 2006). Although these markers are surrogate measures of insulin sensitivity, observed associations between many of these markers and PIH risk further suggest a role for insulin resistance in the development of PIH (Caren & soloman, 2001).

### e. SHBG in PIH

In some studies more direct measurement of insulin sensitivity with a biomarker (sex hormone binding globulin, SHBG) was used to assess insulin resistance. SHBG is a glycoprotein synthesized by the liver that mediates the balance between free (biologically active) and bound (biologically inactive) testosterone and estrogens (Anderson, 1974). Several clinical and *in vitro* studies indicate that $E_2$ and thyroid hormone are the principal stimuli for hepatic SHBG secretion, whereas insulin, PRL, androgens, and GH suppress SHBG (Pasquali et al, 1997; Hampl & Starka, 1996; Krotkiewski, Holm & Shono, 1997; Loukovaara, Carson & Adlercreutz, 1995).

Reduced SHBG is a marker of hyperinsulinemia (Haffner et al, 1988) and insulin resistance (Sherif, Kushner & Falkner, 1998). In studies of nonpregnant women, SHBG levels correlate inversely with glucose tolerance (Goodman-Gruen & Barrett-Connor et al, 1997) and insulin levels (Haffner et al, 1988). The link between SHBG and insulin resistance has important clinical implications. Hyperinsulinemia is known to suppress SHBG synthesis in the liver (Nestler et al, 1991). A low SHBG concentration is considered an independent risk marker for the development of type 2 diabetes in women (Lindstedt et al, 1991; Haffner et al, 1993). There is an inverse relationship between hyperinsulinemia and SHBG concentration (Katsuki et al, 1996; Gascon et al, 2000; Haffner, Dunn & Katz, 1992).

Serum levels of SHBG are relatively high in boys and girls until puberty (Apter et al, 1984; Belgorosky & Rivarola, 1986) and SHBG steroid-binding sites are largely unoccupied during childhood because plasma sex steroid levels are negligible (Gupta, Attanasio & Raaf, 1975). During puberty, serum SHBG levels in boys decrease by approximately one-half, while the amounts of circulating testosterone increase substantially (Gupta, Attanasio & Raaf, 1975). This results in a large reduction in the number of unoccupied SHBG steroid-binding sites in boys as they mature sexually. In girls, there is a much smaller decrease in circulating SHBG during puberty (Apter et al, 1984), and endogenous sex steroid levels are much lower in women than in men. As a result, 80% of the SHBG steroid-binding sites are unoccupied in women versus only 44% in men (Westphal, 1986).

In women, the use of certain oral contraceptives not only markedly reduces the gonadal production of sex steroids, but causes a three to five fold increase in serum SHBG levels (Hammond, 1995) and the vast majority of SHBG steroid-binding sites will be unoccupied under these conditions. Thus, in children and women, and particularly in women taking oral contraceptives, large numbers of SHBG steroid-binding sites are available to bind non-steroidal ligands.

In normal pregnancy, SHBG levels rise steadily during the first and second trimesters, reaching a peak that is 4–6 times the normal nonpregnant range (Kerlan et al, 1994; O'Leary et al, 1991). This early gestation increase in SHBG levels mirrors the contemporaneous increase in $E_2$ levels, which rise almost 20 fold during the first trimester alone (Kerlan et al, 1994; O'Leary et al, 1991). $E_2$ levels continue to rise through the end of pregnancy such that by delivery, levels reach greater than 100 times the normal, nonpregnant, early follicular phase range (Kerlan et al, 1994).

In pregnancy, women with gestational diabetes displayed markedly lower SHBG levels compared with women without gestational diabetes (Bartha et al, 2000). Furthermore, when insulin sensitivity is increased pharmacologically, SHBG levels rise (Crave et al, 1995; Velazquez et al, 1994). Insulin resistance and insulin levels also increase progressively during normal gestation, but the greatest increment occurs during the second half of pregnancy (Catalano et al, 1991; Stanley, Fraser & Bruce, 1998). This physiological increase in insulin resistance during the third trimester may prevent further increases in SHBG levels.

In a third trimester study, women with gestational diabetes were more insulin resistant and had significantly reduced SHBG levels compared with normoglycemic controls despite similar $E_2$ and thyroid hormone levels (Bartha et al, 2000). This sex hormone-binding globulin (SHBG) regulates the access of testosterone and 17b-estradiol ($E_2$) to their target tissues (Hammond, 1995; Siteri et al, 1982).

Polycystic ovary syndrome, which is associated with insulin resistance and low SHBG levels, has been linked to increased risk for pregnancy-induced hypertension (Urman et al, 1997). Insulin is a potent inhibitor of hepatic SHBG synthesis (Plymate et al, 1988) and thus, reduced SHBG is a marker of hyperinsulinemia (Haffner et al, 1988) and insulin resistance (Sherif, Kushner & Falkner, 1998) The clinical utility of SHBG measurement as an index of insulin resistance was established by two large prospective studies, in which reduced baseline SHBG levels were independently associated with future type 2 diabetes (Lindstedt et al,1991; Haffner et al, 1993). A prospective study, reported by (Wolf et al, 2002) association between SHBG and risk of PE was examined in greater detail. It was shown by simple logistic regression model, every 100 nmol/liter rise in serum SHBG was associated with a 31% reduced risk of preeclampsia [odds ratio (OR), 0.69; 95% confidence interval (CI), 0.55, 0.88; $P$=0.01] and in multivariate model, every 100 nmol/liter increase in serum SHBG was independently associated with a 34% reduced risk of preeclampsia (OR, 0.66; 95% CI, 0.47, 0.92; $P$=0.01), which was similar to the unadjusted analysis. It was concluded that reduced first-

trimester SHBG levels were independently associated with increased risk of developing PE (Wolf et al, 2002).

The usual period of onset of PIH (late pregnancy) (Kuhl, 1991) corresponds with the maximal degree of insulin resistance and it supports a possible association. Postulated mechanism through which insulin resistance might increase blood pressure in pregnancy, as in essential hypertensive, include sympathetic nervous activation (Rowe et al, 1981; Reaven et al, 1996) renal sodium retention (DeFronzo et al, 1975), increased cation transport (Doria et al, 1991) and associated endothelial dysfunction (Gibbons et al, 1994). These assumptions, however, do not substitute for more direct evidences on the potential causative role of insulin resistance in the genesis of PIH obtained through prospective data. Such prospective studies are still scanty in number, limited in scope and inconclusive in nature.

Several metabolic derangements, however, precedes PE and some of these persists even after pregnancy is over. These observations suggest that maternal features need to be considered in exploring the etiopathogenesis of the disease. No single cause is likely to explain all cases of hypertension in pregnancy. It is rather more likely that different etiologies may lead to the same phenotype in different groups of women. This realization necessitates the conduction of relevant studies on different racial and environmental background.

## f. Insulin as a causal factor in PIH

Although the association of insulin resistance with PIH is now widely accepted their causal relationship is not yet established. Observations regarding the role of hyperinsulinemia and insulin resistance in the etiopathogenesis of essential hypertension (Yasuhi et al, 2001) have been extrapolated and a similar role of insulinemic abnormalities have been postulated in case of both the disorders constituting PIH (Solomon et al 2001; Kaaja et al,1995; Sattar et al, 2001; Innes, Wimsatt & McDuffie, 2001). However, data in favor of this hypothesis are limited and conflicting. Some studies report an association between insulin resistance and GH but not PE (Caruso et al, 1999; Solomon et al, 1994; Roberts, 1998; Bartha et al, 1997), whereas others report the opposite (Joffe et al, 1998; Solomon et al, 1999; Sowers, Saleh & Sokol, 1995). Most studies that examined insulin resistance in PE and GH, however, were cross-sectional or retrospective (Wolf, 2002), and as a result, it remains unclear whether insulin resistance is involved in the pathogenesis of preeclampsia or is a consequence of the disease.

The longitudinal studies regarding causal relation between insulin resistance and PE are scarce. Only a few two longitudinal studies have been published regarding insulin resistance and PIH, but those studies were limited only in first trimester (Wolf et al, 2002), pregnancy was not followed up till delivery and that was done only in PE.

Midgestation fasting hyperinsulinemia has been found to predict subsequent development of PE in African-American Gravidas (Sowers, Saleh & Sokol, 1995)

and PIH in a Japanese cohort at risk for gestational diabetes (Hamasaki et al, 1996). Another study (Rowe et al, 1981) suggests that amplified midpregnancy β-cell secretory activity (as reflected in fasting and postprandial CP concentrations) is associated with subsequent development of PIH and that this association is independent of obesity and midpregnancy blood pressure. In contrast, others have found that hyperinsulinemia at 26-28 weeks' gestation was not predictive of PIH after controlling for BMI, race and age (Cioffi et al, 1997). To the best of our knowledge only one study has yet addressed the issue of the association of early pregnancy insulin sensitivity with the development of either PE or GH in a later stage (Wolf et al, 2002). Thus there remains a need for more data to determine whether insulin resistance plays a causal role in the development of PE, GH or both (Ellen, Seel & Solomon, 2003).

Accumulating research indicating associations of features of the insulin resistance syndrome and PIH suggests that additional data are needed to elucidate their potential role in pathogenesis, utility for risk stratification, and implications for intervention strategies (Ellen, Seel & Solomon, 2003). Large prospective longitudinal studies are also needed to determine whether there are markers of insulin resistance with sufficient sensitivity and specificity to be clinically relevant. Studies should be undertaken to assess the effects of specific interventions directed at the insulin resistance syndrome on the risk of developing hypertensive pregnancy. An attractive approach for investigating the issue is the study of different indices and markers of insulin resistance to identify the factor(s) involved

in development of PIH. This can provide a deeper insight into the pathophysiology of PIH. The present study was designed with the above prospective in mind.

## Research questions

- What are the frequencies of PIH and its subtypes (PE and GH) in Bangladeshi pregnant subjects in an urban hospital based setting?
- What are the anthropometric and clinical risk factors associated with PIH?
- Which of the indices of insulin resistance may be causally associated with PIH?
- Which of the covariates of insulin resistance may have a causal association with PIH?
- What are the values of early pregnancy insulin sensitivity indices and biomarkers of associated covariates in the prediction of PIH at the later stages of pregnancy?

## Hypothesis

Insulin resistance in early pregnancy is associated with the development of PIH after midpregnancy and indices of early pregnancy insulin resistance and/or biomarkers of its covariates may have a predictive role in PIH.

# Chapter 2

# OBJECTIVES

# 2. OBJECTIVES

## General objectives

The general objective of the study was to investigate the role insulin resistance and its covariates in the etiopathogenesis of pregnancy induced hypertension.

## Specific objectives

- To assess the frequency of PIH and its subtypes (PE and GH) in an urban hospital based Bangladeshi population.
- To study the clinical and anthropometric risk factors associated with PIH.
- To measure the indices of insulin sensitivity at early stages of pregnancy.
- To measure some covariates of insulin resistance at early stages of pregnancy.
- To explore the association of early pregnancy insulin resistance and its covariates with the development of PIH in later stages of pregnancy.
- To investigate the predictive roles of early pregnancy indices of insulin resistance and biomarkers of its covariates in the detection of PIH in later stages of pregnancy.

# 3. SUBJECTS AND METHODS

## Study design

It was a prospective study with a nested case-control design; the pregnant women were followed from early pregnancy up to delivery. The design also allowed outcome (PIH) analysis in terms of frequency as in a cross-sectional survey.

## Study place and duration

The study was conducted in the Biomedical Research Group of BIRDEM with subjects collected from various public hospitals in Dhaka city. It was carried out during the period of October 2004 to January 2008.

## Study Subjects

A number of 430 subjects, attending the out-patient departments of various public hospitals in the Dhaka city for antenatal care, were enrolled in the study and out of them 404 could be followed up until the end of their pregnancies in the study period.

## Inclusion criteria

Healthy pregnant women with normal singleton pregnancies (gestation period 8-16 wks) without having any medical disorder of pregnancy.

## Exclusion criteria

- Pregnancy with diabetes mellitus
- Pregnancy with chronic hypertension
- Pregnancy with chronic renal disease
- Multiple pregnancies

- Taking antifolate drugs (antiepileptics)
- Smokers who are pregnant
- Pregnancy with other medical diseases
- Pregnant women who were alcoholic.

**Sample size**

Keeping the cross-sectional survey component of the design (for exploring the prevalence of PIH) the total number of pregnancies was calculated using the following formula (Blant, 2002).

$$n = \frac{p(1-p)}{SE^2}$$

Were, n is the number of subjects; p is the unknown population and SE is the standard error of mean. Assuming the approximate population of PIH (from various international reports) as 10% in the pregnant women and taking the 95% confidence interval to be 0.05 on either side (ie SE as 0.025, the number of samples (pregnant mother) became 360.

For the case-control part of the study the sample size was calculated by the following formula (Hennekens & Buring, 1987).

n (each group)= $(p_0q_0 + p_1q_1)(z_1\text{-}\alpha/2 + z_1\text{-}\beta)^2/ (p_1\text{-}p_0)^2$

Where $p_1$= the postulated proportion of insulin resistance in cases

$p_0$= the postulates proportion of insulin resistance in controls

$q_1 = 1\text{-}p_1$

$q_0 = 1\text{-}p_0$

$z_1\text{-}\alpha/2$ = value of the standard normal distribution corresponding to a significance level of alpha (eg, 1.96 for a two sided test at the 0.05 level).

$z_1$- $\beta$ = Value of the standard normal distribution corresponding to the desired level of power (eg, 0.84 for a power of 80%).

In the absence of any background studies on insulin resistance in the early pregnancy we used the proportionate losses of insulin sensitivity in Control, PE and GH (15%, 53% and 47% respectively) as found in a study on the same popuiation[30], as a guide to calculate the sample size (with rounding of the figures for PE and GH to 50%).

Thus, for the present proposal

$p_0$= 0.15; $p_1$= 0.50

$q_0$ = 1-$P_0$ = 1- 0.15 = 0.85; $q_1$= 1-$p_0$ = 1-0.50 = 0.50

$z_1$- $\alpha/2$= 1.96; $z_1$- $\beta$ = 0.84

Putting the values in the equation:

$$n \text{ (each group)} = \frac{[(0.15)(0.85)+ (0.50)(0.50)][1.96+0.84]^2}{(0.50- 0.15)^2}$$

$$= \frac{[0.1275+0.2500](7.84)}{0.1225}$$

$$= \frac{(0.3775)(7.84)}{0.1225}$$

$$= \frac{2.9596}{0.1225}$$

$$=24$$

Thus, with 80% power and 5% level of significance the minimum number for each of the Control, PE and GH groups would be 24. To be on the safer side we have decided to include around 40 cases of PE and 40 cases of GH. Assuming, from published literatures in other populations of developing countries, we can guess that PE complicates 10% of pregnancies and a similar

percentage is complicated by GH. Thus an initial recruitment of 400 pregnant subjects was required for the project.

Collection of subjects and division of Cases and Controls. A total of 430 pregnant women were recruited for in the study but 26 could not be followed up until the end of their pregnancy. Thus a total number of 404 subjects participated in the study. The exact nature and purpose of the study was explained to the pregnant women who registered at the hospitals during the period of the study. Only those pregnant women who gave informed consent (as per proforma given in Appendix-I) were included in the study. A total of 26 women could not be followed up until their end of pregnancy. First the PIH and Non-PIH cases were analyzed. Then, by a nested case-control design, women matched with PIH cases for age and gestational age were selected as Control (n=101).

## Development of Questionnaire

A questionnaire was developed to obtain relevant information of demographic and socioeconomic data such as age, educational status, occupational status and the obstetric history like para, gravida, and previous obstetric history. The questionnaire also included anthropometric data, drug and medical history and clinical information. The questionnaire was coded and pre tested before finalization. The questionnaire was both closed and open ended.

## Collection of data

After taking informed consent relevant data and fasting blood and urine samples were collected from volunteers who met the selection criteria of the study subjects. Detailed sociodemographic data, family history and medical history were recorded on a pre-designed data collection sheet. All interviews were conducted in the hospital. Physical examination was done and anthropometric measurements (height, weight) of each subject were taken and recorded. Obstetric examination was performed and recorded for every patient. All the patients were followed up monthly for development of hypertension and this was continued until delivery. For collection of blood and urine the patients were referred to the Biomedical Research Group, BIRDEM.

## Anthropometric Data

### Weight

Body weight was measured on a lever balance (Detecto-Medic, Detecto Scales, Inc, USA). The balance was calibrated every day before use. The body weight was measured bare footed to the nearest 0.1 kg with clothes on. The average weight (0.5 kg) of the clothes was later subtracted from the measured weight. The measurement of weight was done after the bladder has been emptied, and before a meal.

### Height

Heights of the subjects were measured barefooted in the standing position with a stander scale to the nearest 0.1 cm (Detecto-Medic, Detecto Scale Inc, USA).

During measuring height some precautions were taken. When measuring

height, the subjects stood straight with the head positioned such that the Frankfurt plane is horizontal, feet together, knees straight, and heels, buttocks and shoulder blades in contact with the vertical surface of the stadiometer.

**Body Mass Index (BMI)**

Body mass index were calculated from the body weight and height of the subjects using the following formula weight in kg divided by height in meter square.

$$BMI = \frac{\text{Weight in kg}}{(\text{Height in meter})^2}$$

**Measurement of Blood Pressure as per ACOG** (Fernando,1993)

BP was measured with the patient in lying position keeping sphygmomanometer at the level of the heart. When DBP was found more than 90 mm of Hg, it was confirmed on two different occasions at least 6 hours apart (point of muffling i.e. K IV).

**Criteria of PIH, PE and GH**

Hypertension in pregnancy includes the following disorders as per definitions from Solomon & Seely (2001):

Pregnancy Induced Hypertension (PIH): Gestational Hypertension (GH) and Preeclampsia (PE) are together considered as pregnancy induced hypertension or PIH.

- Gestational Hypertension (GH): Blood pressure elevation detected for

the first time after midpregnancy and distinguished from PE by the absence of proteinuria (non-proteinuric hypertesion).

- Preeclampsia (PE): Hypertension developing after 20 weeks of gestation accompanied by significant proteinuria (protenuric hypertension).

Hypertension was defined following the criteria of the American College of Obstetrics and Gynecology (ACOG) by Fernando (1992). According to ACOG Hypertension is defined as:

BP equal to or greater than 140/90 mm of Hg, rise of systolic BP 30 mm of Hg and rise of diastolic BP (Point of muffling i.e. K IV) 15 mm of Hg or more.

Proteinuria was initially diagnosed by on dipstick test and collected urine was analyzed by Quantitive estimation of ratio between protein and creatinine.

**Collection of Blood and Urine Sample**

Subjects were requested to fast overnight (12 hours) and not to smoke or take any kind of medicine on the previous day. They were then requested to attend the Biomedical Research Group of BIRDEM on the next morning. Blood samples were collected, following all aseptic precautions, from the ante-cubital vein using disposable plastic syringe. 10 cc of blood was transformed in a haperinazed test tube. Plasma was separated by centrifugation (10 minutes) at a rate of 150g (2000 rpm) at room temperature immediately after the blood was allowed to clot for 30 minutes. Separated Plasma was aliquoted and preserved immediately at -27$^0$C for the future estimation of the following biochemical analysis:

Fasting glucose, TG, total cholesterol, HDL cholesterol, LDL cholesterol, creatinine, insulin and Sex Hormone Binding Globulin (SHBG).

Clean catch fresh morning urinary sample (5ml) were collected in a clean container and assayed for protein. Urine samples were centrifuged and kept at $-27^0$C until analysis for protein-creatinine ratio.

**Laboratory Methods**

- Glycemic status of the study subjects was measured by OGTT fasting plasma glucose load using Glucose Oxidase method (Randox, UK) (Appendix-III)
- Lipidemic status was assessed by
    - Plasma total cholesterol by enzymatic endpoint method (cholesterol Oxidase/ Peroxidase) (Randox Laboratories, UK) (Appendix-IV)
    - Plasma triglyceride by enzymatic-colorimetric (GPO-PAP) method (Randox laboratories, UK) (Appendix-V)
    - Plasma high density lipoprotein (HDL) by enzymatic-colorimetric (cholesterol CHOD-PAP) method (RANDOX laboratories, UK) (Appendix-VI)
    - The LDL-cholesterol level in plasma was calculated by using Friedewald's formula. (Appendix-VII)
- Serum Insulin level was estimated by Microparticle Enzyme Immunoassay (MEIA) technology. (Appendix-VIII)
- Serum SHBG was estimated (Sex Hormone Binding Globulin) was estimated by Chemiluminescent Immunometric Assay. (Appendix-IX)
- Serum creatinine was estimated by alkaline-picrate methods (Randox Laboratories, UK). (Appendix-X)

- Urine total protein was measured by Pyrogallol red method (QCA, Spain). (Appendix-XI)
- Urinary protein (albumin) was measured by Uriscan Strip (YD Diagnostic, Thailand). (Appendix-XII)
- Urine Creatinine by alkaline-picrate methods (Randox Laboratories, UK). (Appendix-XIII)
- Insulin secretory capacity (HOMA B%) and insulin sensitivity (HOMA S%) were assessed by Homeostasis Model Assessment (HOMA). (Appendix-XIV)
- Quantitative insulin sensitivity check index (QUICKI). (Appendix-XV)
- Estimation of UPr/Cr ratio. (Appendix-XVI).

**Indices of Insulin Sensitivity:**

Glucose Insulin ratio; Quantitive Insulin Sensitivity Check Index (QUICKI); Insulin secretory Capacity (HOMA%B) and Insulin sensitivity (HOMA%S) (McAuley, 2001).

**Biomarkers of endothelial dysfunction:**

Urine protein creatinine ratio (UPr/Cr) (Elhadd et al, 2001).

## STATISTICAL ANALYSIS

Statistical analysis was performed with the SPSS V.12 (SPSS Inc, Chicago, IL, USA). Data were expressed as mean±SD for parametric values and median (range) for non-parametric values.

Comparisons between groups were done using Mann-Whitney U test for skewed data and Independent t-test for normally distributed data.

The relationships between the variables were explored by bivariate as well as multivariate analysis. To test the association between two variables, spearman's coefficient correlation was done for non-normally distributed data and Pearson's correlation for normally distributed data.

Logistic regression analysis was done to test the association of the discrete dependent variables adjusting the effect of independent variable. For continuous dependent variables multiple linear analysis was performed.

Sensitivity, specificity, positive and negative predictive values were calculated by McNemar test and by several cut-off values of a parameter, and ROC curve was constituted to determine an optimal value that maximized sensitivity and specificity for a predictor. A probability value of $<0.05$ was considered significant.

# Chapter 4

# RESULTS

# 4. RESULTS

## Baseline characteristics of the study subjects (Table 1)

Out of 430 pregnant subjects enrolled in this study, a total number of 404 could be followed up to the delivery and baseline characteristics of these subjects were analyzed.

**Table 1**: Baseline characteristics of the study subjects (n=404)

| Characteristics | |
|---|---|
| Age (years) | 25±4.36 |
| BMI (kg/m$^2$) | 22.4±4.07 |
| Gestational weeks at 1$^{st}$ visit (wks) | 13 (4-32) |
| Parity | |
| Nullipara | 271 (67%) |
| Multipara | 133 (33%) |
| Positive family history of hypertension | 212 (52%) |
| Positive history of gestational hypertension in previous Pregnancy | 235 (58%) |
| Positive history of PE in previous pregnancy | 240 (59%) |
| Positive history of drug (Folate) intake | 213 (53%) |
| SBP (mm Hg) | 108±11.3 |
| DBP (mm Hg) | 69.7±8.05 |
| MBP(mm Hg) | 82.7±8 |
| F glucose (mmol/l) | 4.4±0.7 |
| 2hrs after 75gm glucose (mmol/l) | 6.8±1.8 |
| TG (mg/dl) | 120 (42-423) |
| Cholesterol (mg/dl) | 175 (104-310) |
| HDL (mg/dl) | 52 (30-80) |
| LDL (mg/dl) | 105 (23-233) |
| Serum creatinine (mg/dl) | 0.86 (0.09-1.8) |

*Results are expressed as Mean±SD , number (%) or median (range) as appropriate, n= number of subjects. BMI=Body Mass Index, SBP=Systolic Blood pressure, DBP=Diastolic Blood Pressure, MBP=Mean Blood pressure, TG=Tri-Glyceride, HDL=High Density Lipoprotein, LDL=Low Density Lipoprotein.*

The characteristics (Table 1) show that the subjects were fairly young (mostly below 30 yrs), normal weight or only mildly overweight, mostly nulliparous (67%) and had normoglycemia, normolipidemia and normal kidney function.

### Proportion of PIH (PE and GH) cases (Table 2)

At the end of the follow up of 404 cases, 41 pregnant women were found to develop Pregnancy Induced Hypertension (PIH); among them 10 pregnant women developed PE and 31 developed GH. Thus, the frequency of PIH was found to be 10.14% (PE 2.47% and GH 7.67%).

**Table 2:** Proportion of PIH (PE and GH) cases at the end of follow up

| | |
|---|---|
| Number of cases followed | 404 (100 %) |
| Number (%) of PIH cases | 41(10.14%) |
| Number (%) of PE cases | 10 (2.47%) |
| Number (%) of GH cases | 31 (7.67%) |

*Results are expressed as number (%) distribution.*

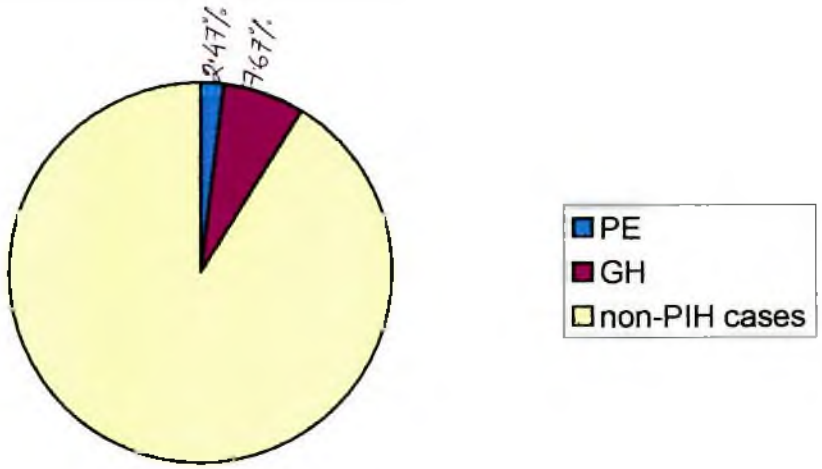


**Figure 1:** Proportion of PIH (PE and GH) cases among the pregnant subjects

## Baseline characteristics of non-PIH and PIH subjects (Table 3)

The age (yrs, mean±SD) of the groups were as follows: non-PIH 26±3.3 and PIH 26 ±3.6.The groups were found to be matched to each other regarding age.

**Table 3:** Baseline characteristics of non-PIH and PIH subjects (n=404)

| **Characteristics** | **Non-PIH** (n=363) | **PIH** (n=41) | ***P Value*** |
|---|---|---|---|
| Age (years) | 26±3.3 | 26±3.6 | 0.210 |
| BMI (kg/m$^2$) | 21±4.04 | 23.1±4.13 | 0.078 |
| Gestational weeks at 1$^{st}$ visit (wks) | 13±4.3 | 13.05±3 | 0.860 |
| Parity | | | |
| Nullipara | 251(69%) | 20(49%) | 0.027 |
| Multipara | 111(31%) | 21(51%) | |
| Positive family history of hypertension | 187(52%) | 25(61%) | 0.250 |
| Positive history of gestational hypertension in previous pregnancy | 208(57%) | 27(66%) | 0.293 |
| Positive history of PE in previous pregnancy | 220(61%) | 20(49%) | 0.144 |
| Positive history of drug (Folate) intake | 190(52%) | 23(56%) | 0.648 |
| SBP (mm Hg) | 108±11.6 | 110±7.9 | 0.494 |
| DBP (mm Hg) | 69±8.15 | 70±6.8 | 0.054 |
| MBP (mm Hg) | 83.3±8.2 | 84±6.01 | 0.110 |
| F glucose (mmol/l) | 4.4±0.6 | 4.34±0.91 | 0.466 |
| 2hrs after 75gm glucose (mmol/l) | 6.6±1.7 | 6.9±2.5 | 0.138 |
| Cholesterol (mg/dl) | 176(104-310) | 47(31-75) | 0.065 |
| TG (mg/dl) | 122(40-423) | 105(50-296) | 0.620 |
| HDL (mg/dl) | 52(30-80) | 47(31-75) | 0.701 |
| LDL (mg/dl) | 107(23.8-233) | 100(45-171) | 0.071 |
| Serum creatinine (mg/dl) | 0.86(0.9-1.8) | 0.88(0.74-1.05) | 0.470 |

*Results are expressed as Mean±SD, number (%) and median (range) as appropriate; n= number of subjects; Independent t-test, Mann-Whitney U test and chi-square test were done as tests of significance, according the nature and distribution of variables. BMI=Body Mass Index, SBP=Systolic Blood pressure, DBP=Diastolic Blood Pressure, MBP=Mean Blood pressure, TG=Tri-Glyceride, HDL=High Density Lipoprotein, LDL=Low Density Lipoprotein.*

In the non-PIH group (n=363) 69% of the study subjects were nulliparous, 31% were multiparous. In PIH cases (n=41) 49% were nulliparous and 51% were multiparous.

Regarding positive family history of hypertension, 187 non-PIH pregnant mothers out of 363 (52%) and 25 (61%) out of 41 were found to have hypertension. 208 (57%) in non-PIH and 27 (66%) in PIH subjects showed history of gestational hypertension in previous pregnancy. There was no significant difference between the groups in this regard.

History of PE in previous pregnancy was positive in 220 (61%) non-PIH and 20 (49%) PIH subjects. There was a history of taking oral folate 192 (52%) in non-PIH and 23 (56%) in PIH subjects.

The diastolic blood pressure (mm Hg, M±SD) during first trimester were as follows: non-PIH 69±8.15, PIH 70±6.8; (p=0.054). There was a marginally significant difference in the diastolic blood pressure (mm Hg) between the two groups during their first trimester.

**Frequency of PIH in Nullipara and Multipara subjects (Table 4)**

Out of 404 cases, 271 cases were nullipara and 132 were multipara. Among nulliparous women 20(7.4%) developed PIH and 251(92.6%) cases were Non-PIH. Among multiparous women 21(15.9%) developed PIH and 111(84.1%) were non-PIH. Both the gropus showed significant difference.

**Table 4:** Frequency of PIH in Nullipara and Multipara subjects (n=404)

| Groups | Nullipara n (%) | Multipara n (%) | *P value* |
|---|---|---|---|
| PIH | 20(7.4%) | 21(15.9%) | |
| Non-PIH | 251(92.6%) | 111(84.1%) | 0.027 |
| Total | 271(100%) | 132(100%) | |

*Results are expressed as number (%) distribution. The significant of difference between the groups was calculated by chi-square test.*

**Association of PIH with various baseline parameters as explored by binary logistic regression. (Table 5)**

Binary logistic regression analysis with PIH as a dependent variable and parity, BMI, DBP, MBP and Creatinine as independent variable, shows a highly significant association of PIH with only parity after adjustment of the effects of BMI, DBP, MBP and Creatinine.

**Table 5:** Association of PIH with various baseline parameters as explored by binary logistic regression (n=404)

| Variables | β | *SE.* | *p* |
|---|---|---|---|
| Parity | 0.705 | 0.217 | 0.001 |
| BMI ($kg/m^2$) | 0.036 | 0.043 | 0.399 |
| DBP (mm of Hg) | 0.032 | 0.021 | 0.132 |
| MBP (mmHg) | 0.023 | 0.022 | 0.295 |
| Creatinine (mg/dl) | 1.143 | 1.715 | 0.505 |

*β for standardized regression coefficient. PIH was taken as dependent variable whereas other variables were taken as independent variable; n = number of subjects,*

**Association of SBP and DBP at 1st trimester as (dependent variable) with other baseline parameters as explored by multiple regression (Table 6)**

Multiple regression analysis of Systolic blood pressure ($SBP_1$) at 1st visit as a dependent variable (DV) with other variable, where a significant negative association was found between SBP with gestational week (β=-0.11; p=0.021), BMI and HDL has got also positive significant association with SBP (β=0.266; p=<0.001 and β=0.196;p=<0.001).

**Table 6:** Association of SBP and DBP at 1st trimester as (dependent variable) with other baseline parameters as explored by multiple regression (n=404)

| Characteristics | SBP at 1st visit | | DBP at 1st visit | |
|---|---|---|---|---|
| | β | P | β | p |
| Age (yrs) | 0.089 | 0.076 | 0.129 | 0.012 |
| Parity | 0.024 | 0.621 | -0.052 | 0.297 |
| Gestational week | -0.11 | 0.021 | 0.062 | 0.199 |
| Positive family history of hypertension | -0.057 | 0.231 | 0.022 | 0.645 |
| Positive history of GH in previous pregnancy | 0.015 | 0.747 | 0.006 | 0.899 |
| Positive history of PE in previous pregnancy | -0.022 | 0.642 | 0.025 | 0.599 |
| Positive history of drug (Folate) intake | -0.006 | 0.896 | -0.001 | 0.978 |
| BMI ($kg/m^2$) | 0.266 | <0.001 | 0.278 | <0.001 |
| F glucose (mmol/L) | 0.078 | 0.100 | 0.065 | 0.177 |
| 2 hrs after 75 gm glucose level | 0.067 | 0.171 | 0.063 | 0.204 |
| Triglyceride (mg/dl) | 0.001 | 0.980 | -0.031 | 0.565 |
| Cholesterol (mg/dl) | -0.022 | 0.671 | -0.110 | 0.038 |
| HDL (mg/dl) | 0.196 | <0.001 | 0.086 | 0.073 |
| Serum creatinine (mg/dl) | -0.040 | 0.410 | -0.040 | 0.323 |

*β for standardized regression coefficient. Systolic blood pressure and Diastolic blood pressure was taken as dependent variable whereas others were taken as independent variable; n=number of subjects, F glucose=Fasting glucose; HDL=High density of lipoprotein.*

Diastolic blood pressure ($DBP_1$) at 1st visit as a dependent variable (DV) with other variable, where a significant negative association was found between DBP with cholesterol (β=-0.110;p=0.038), Age and BMI has got also positive significant association with SBP (β=0.129; p=0.012 and β=0.278;p=<0.001).

**Association of SBP and DBP at 2nd trimester as (dependent variable) with other baseline parameters as explored by multiple regression (Table 7)**

**Table 7:** Association of SBP and DBP at 2nd trimester as (dependent variable) with other baseline parameters as explored by multiple regression (n=404)

| Characteristics | SBP at 2nd visit | | DBP at 2nd visit | |
|---|---|---|---|---|
| | β | P | β | p |
| Age (Yrs) | 0.054 | 0.309 | 0.015 | 0.778 |
| Parity | 0.037 | 0.475 | 0.029 | 0.581 |
| Gestational Wk | 0.026 | 0.627 | 0.020 | 0.690 |
| Positive family history of hypertension | -0.121 | 0.016 | -0.096 | 0.056 |
| Positive history of GH in previous pregnancy | -0.028 | 0.577 | -0.055 | 0.279 |
| Positive history of PE in previous pregnancy | 0.062 | 0.221 | 0.027 | 0.595 |
| Positive history of drug (Folate) intake | 0.085 | 0.097 | 0.061 | 0.232 |
| BMI ($kg/m^2$) | -0.020 | 0.708 | -0.006 | 0.904 |
| F Glucose (mmol/L) | 0.042 | 0.401 | 0.058 | 0.251 |
| 2 hr after 75 gm glucose level | 0.056 | 0.282 | 0.012 | 0.823 |
| Triglyceride (mg/dl) | -0.068 | 0.228 | -0.073 | 0.197 |
| Cholesterol (mg/dl) | 0.085 | 0.123 | 0.095 | 0.086 |
| HDL (mg/dl) | -0.062 | 0.214 | -0.048. | 0.341 |
| Serum creatinine (mg/dl) | -0.085 | 0.099 | -0.085 | 0.101 |

*β for standardized regression coefficient. SHBG=Sex hormone binding globulin, rate was taken as dependent variable whereas others were taken as independent variable; n=number of subjects, MBP=mean blood pressure; F Glucose=Fasting glucose; HDL=High Density of lipoprotein, HOMA%S=Value of Insulin sensitivity by Homeostasis Model Assessment.*

Multiple regression analysis of Systolic blood pressure ($SBP_2$) at 2nd visit as a dependent variable (DV) with other variable, where a significant negative association was found positive family history of hypertension (β=-0.121; p=0.016).

**Table 8:** Association of SBP and DBP at 3rd trimester as (dependent variable) with other baseline parameters as explored by multiple regression (n=404)

| Characteristics | SBP at 3rd visit | | DBP at 3rd visit | |
|---|---|---|---|---|
| | β | P | β | p |
| Age (yrs) | -0.075 | 0.160 | -0.015 | 0.785 |
| Parity | 0.003 | 0.959 | -0.034 | 0.521 |
| Gestational week | 0.065 | 0.205 | 0.018 | 0.723 |
| Positive family history of hypertension | 0.152 | 0.003 | 0.023 | 0.652 |
| Positive history of GH in previous pregnancy | 0.024 | 0.636 | -0.012 | 0.813 |
| Positive history of PE in previous pregnancy | -0.004 | 0.940 | 0.022 | 0.672 |
| Positive history of drug (Folate) intake | -0.023 | 0.653 | 0.016 | 0.761 |
| BMI ($kg/m^2$) | -0.032 | 0.547 | -0.067 | 0.209 |
| F glucose (mmol/L) | 0.047 | 0.346 | 0.068 | 0.183 |
| 2 hr after 75 gm glucose level | -0.009 | 0.868 | -0.042 | 0.424 |
| Triglyceride (mg/dl) | -0.003 | 0.962 | -0.032 | 0.571 |
| Cholesterol (mg/dl) | 0.007 | 0.897 | 0.035 | 0.532 |
| HDL (mg/dl) | -0.056 | 0.271 | 0.114 | 0.025 |
| Serum creatinine (mg/dl) | 0.008 | 0.880 | 0.040 | 0.443 |

*β for standardized regression coefficient. SHBG=Sex hormone binding globulin, rate was taken as dependent variable whereas others were taken as independent variable; n=number of subjects, MBP=mean blood pressure; F Glucose=Fasting glucose; HDL=High Density of lipoprotein, HOMA%S=Value of insulin sensitivity by Homeostasis Model Assessment.*

**Association of SBP and DBP at 3rd trimester as (dependent variable) with other baseline parameters as explored by multiple regression (Table 8)**

Multiple regression analysis of Systolic blood pressure ($SBP_3$) at 3rd visit as a dependent variable (DV) with other variable, where a significant positive association was found positive family history of hypertension (β=0.152; p=0.003).

**Baseline characteristics of the Control and PIH subjects (Table 9)**

The BMI (kg/m$^2$; mean±SD) were significantly higher in PIH subjects (23.7±4.1) as compared to Control (22.4±3.4); (p=0.047).

**Table 9:** Baseline characteristics of the Control and PIH subjects (n=142)

| **Characteristics** | **Control** (n=101) | **PIH** (n=41) | ***P Value*** |
|---|---|---|---|
| Age (years) | 26±3.1 | 26±3.6 | 0.109 |
| BMI (kg/m$^2$) | 22.4±3.4 | 23.7±4.1 | 0.047 |
| Gestational weeks at 1st visit (wks) | 12.7±2.5 | 13.05±3.07 | 0.573 |
| Parity | 1.6±0.5 | 1.5±0.5 | 0.771 |
| Nullipara | 43 (43%) | 19 (46%) | 0.001 |
| Multipara | 58 (57%) | 22 (54%) | 0.001 |
| Positive family history of hypertension | 43 (43%) | 23 (56%) | 0.143 |
| Positive history of gestational hypertension in previous pregnancy | 32 (32%) | 29 (71%) | <0.001 |
| Positive history of PE in previous pregnancy | 21 (21%) | 11 (27%) | 0.435 |
| Positive history of drug (Folate) intake | 76 (76%) | 32 (78%) | 0.723 |
| $SBP_1$ (mm Hg) | 109±10.7 | 110±7.9 | 0.472 |
| $DBP_1$ (mm Hg) | 69.3±8 | 72±6.8 | 0.057 |
| $MBP_1$ | 82.5±7.7 | 84.8±6 | 0.071 |

*Results are expressed as Mean±SD, number (%) and median (range) as appropriate; n= number of subjects; Independent t-test, Mann-Whitney U test and chi-square test were done as tests of significance, according the nature and distribution of variables. BMI=Body Mass Index, $SBP_1$=Systolic Blood pressure, $DBP_1$=Diastolic Blood Pressure, $MBP_1$=Mean Blood pressure.*

In the control groups (n=101) 43% of the study subjects were nulliparous, 57% were multiparous, in PIH cases (n=41) 46% were nulliparous and 54% were multiparous.

Regarding positive family history of hypertension, 43 pregnant mothers out of 101 (43%) and 23 (56%) out of 41 were found to have hypertension.

32 (32%) in control and 29 (71%) in PIH subjects showed history of gestational hypertension in previous pregnancy. There was highly significant difference between the groups in this regard.

History of PE in previous pregnancy was positive in 21 (21%) in control and 11 (27%) PIH subjects. There was a history of taking oral folate 76 (76%) in control and 78 (78%) in PIH subjects.

A marginally significant difference could be observed in Diastolic blood pressure (DBP) (mm Hg, mean±SD) at first visit between the two groups. In control (69.3±8) and PIH in (72±6.8); (p=0.057).

**Table: 10** Blood pressure in follow up visit (n=142)

| **Variable** | **Control** (n=101) | **PIH** (n=41) | ***P Value*** |
|---|---|---|---|
| $SBP_1$ (mm Hg) | 109±10.7 | 110±7.9 | 0.472 |
| $DBP_1$ (mm Hg) | 69.3±8 | 72±6.8 | 0.057 |
| $MBP_1$ (mm Hg) | 82.5±7.7 | 84.8±6 | 0.071 |
| $SBP_2$ (mm Hg) | 106.5±10.7 | 125.4±22.4 | <0.001 |
| $DBP_2$ (mm Hg) | 68.1±8.7 | 82.8±15.4 | <0.001 |
| $MBP_2$ (mm Hg) | 80.9±56.6 | 96.9±17.4 | <0.001 |
| $SBP_3$ (mm Hg) | 105.4±9.4 | 142.9±20 | <0.001 |
| $DBP_3$ (mm Hg) | 69±8.4 | 98.3±8.1 | <0.001 |
| $MBP_3$ (mm Hg) | 151.4±12.2 | 208.5±20.4 | <0.001 |

*Results are expressed as Mean±SD n= number of subjects; Independent t-test, was done as tests of significance,$SBP_1$=Systolic Blood pressure, $DBP_1$=Diastoiic Blood Pressure, $MBP_1$=Mean Blood pressure, $SBP_2$=Systolic Blood pressure, $DBP_2$=Diastolic Blood Pressure, $MBP_2$=Mean Blood pressure, $SBP_3$=Systolic Blood pressure, $DBP_3$=Dlastolic Blood Pressure, $MBP_3$=Mean Blood pressure.*

## Blood pressure in follow up visit (Table 10)

There was highly significant difference (mm Hg, mean±SD) of SBP [in control (106.5±10.7), in PIH (125.4±22.4), (p=<0.001)]; DBP (mm Hg, mean±SD) [in control (68.1±8.7) and in PIH (82.8±15.4), (p=<0.001)] and MBP (mm Hg, mean±SD) [in control (80.9±56.6) and in PIH (96.9±17.4), (p=<0.001)] at 2nd visit between control and PIH groups respectively.

Similar result shows at 3rd visit SBP [in control (105.4±9.4) and in PIH (142.9±20), (p=<0.001)]; DBP [in control (69±8.4), in PIH (98.3±8.1),(p=<0.001)] and MBP [in control (151.4±12.2), PIH (208.5±20.4), (p=<0.001)] between the two groups.

## Baseline nutritional intake of the study subjects (Table 11)

Iron intake was significantly lower in PIH groups than control. [Dietary iron mg/day, median (range) Control 19.9(10.1-4.3) and PIH 18.8(9.97-30.5); (p=0.051)]. PIH had lower intake of Vitamin $B_2$ and the difference was nearly significant [Control 0.85 (0.30-2.13) and PIH 0.73 (0.21-2.13); (p=0.059)].

**Table 11:** Baseline nutritional intake of the study subjects (n=142)

| **Variable** | **Control** (n=101) | **PIH** (n=41) | ***P Value*** |
|---|---|---|---|
| Total intake of Energy (Kcal/day) | 1537.5 (1011-2646) | 1440.5 (1035.4-2405.4) | 0.206 |
| Total intake of Fiber (gm/day) | 3.62 (0.53-18.15) | 28.4 (13.6-56.1) | 0.352 |
| Total intake of CHO (gm/day) | 246.5 (133.1-495.5) | 229 (133-405.6) | 0.169 |
| Total intake of Protein (gm/day) | 59.8 (25.6-118.1) | 55.2 (25.6-118.1) | 0.618 |
| Total intake of Fat (gm/day) | 30 (11.21-56.1) | 28.4 (13.6-56.1) | 0.810 |
| Total intake of Calcium (mg/day) | 493.6 (71.3-16.26) | 389 (71.3-1346) | 0.165 |
| Total intake of Iron (mg/day) | 19.9 (10.1-44.3) | 18.8 (9.97-30.5) | 0.051 |
| Total intake of Folate (mg/day) | 0.30 (0.10-0.80) | 0.30 (0.10-0.80) | 0.926 |
| Total intake of vitamin $B_1$ (mg/day) | 1.06 (0.49-2.14) | 0.98 (0.51-2.14) | 0.385 |
| Total intake of vitamin $B_2$ (mg/day) | 0.85 (0.30-2.13) | 0.73 (0.21-2.13) | 0.059 |
| Total intake of Vitamin $B_{12}$ (µg/day) | 3.5(1.2-6) | 4(2.2-6) | 0.820 |
| Total intake of Vitamin C (mg/day) | 55.39 (4.72-490) | 46 (9.3-490) | 0.420 |

*Results were expressed as Mean±SD, n=number of subjects; Mann-Whitney U test was done as a appropriate test of significance.*

**Biochemical characteristic of the Control and PIH subjects (Table 12)**

Urine protein-creatinine ratio (UPr/Cr) showed highly significant difference between Control and PIH groups. The [median (range)] of Upr/Cr were in control 4.39 (1.1-15.5) and in PIH groups 9.6 (3.48-20.5); p=<0.001.

**Table 12:** Biochemical characteristic of the Control and PIH subjects (n=142)

| **Variable** | **Control** (n=101) | **PIH** (n=41) | ***P Value*** |
|---|---|---|---|
| F Glucose (mmol/l) | 4.3±0.56 | 4.1±0.54 | 0.081 |
| 2hrs after 75gm glucose (mmol/l) | 6 (3.3-10.7) | 6.5 (3-7.7) | 0.482 |
| TG (mg/dl) | 120.7±36.6 | 128.82±37.9 | 0.240 |
| Cholesterol (mg/dl) | 183.17±36.1 | 179.4±33.84 | 0.575 |
| HDL (mg/dl) | 44.17±10.8 | 42.5±8.58 | 0.337 |
| LDL (mg/dl) | 115.1±30.9 | 110.4±33.08 | 0.419 |
| Serum creatinine (mg/dl) | 0.87±0.1 | 0.91±0.16 | 0.189 |
| UPr/Cr ratio (mg/mmol) | 4.39 (1.1-15.5) | 9.6 (3.48-20.5) | <0.001 |

*Results are expressed as Mean±SD and median (range) as appropriate; n= number of subjects; Independent t-test and Mann-Whitney U test were done as tests of significance, according the nature and distribution of variables. F Glucose=Fasting Glucose, ABF= 2hour after 75gm glucose test, TG=Tri - Glyceride, HDL=High Density Lipoprotein, LDL=Low Density Lipoprotein, Upr/Cr ratio, Urine protein Creatinine ratio.*

**Insulinemic status of the study subjects (Table 13)**

There was nearly significant difference in Insulin level [(uU/ml), median (range)] between control and PIH groups. Insulin level in control and PIH groups were as follows: Control 5.2 (3-9.7) and PIH 6 (3.3-8.6); (p=0.079).

There was significant difference in Glucose/Insulin ratio [median (range)] and the values were as follows: in control 0.80 (0.39-1.67) and in PIH 0.69 (0.44-1.30); (p=0.004).

**Table 13:** Insulinemic status of the study subjects (n=142)

| **Variable** | **Control** (n=101) | **PIH** (n=41) | ***P Value*** |
|---|---|---|---|
| Insulin (μU/ml) | 5.2 (3-9.7) | 6 (3.3-8.6) | 0.079 |
| Glucose-Insulin ratio | 0.80 (0.39-1.67) | 0.69 (0.44-1.30) | 0.004 |
| QUICKI | 2.01 (1.59-2.79) | 1.90 1.64-2.48) | 0.002 |
| HOMA%B | 101 (36.2-281.6) | 166 (85-280) | <0.001 |
| HOMA%S | 155.9 (56.3-418.6) | 110 (51-243) | <0.001 |
| SHBG (nmol/L) | 180 (103-219) | 170 (10-198) | 0.003 |

*Results are expressed as Mean±SD, and median (range) as appropriate; n= number of subjects; Independent t-test and Mann-Whitney U test were done as tests of significance, according the nature and distribution of variables. HOMA %B, Insulin secretory capacity by Homeostasis Model Assessment; HOMA %S, Insulin Sensitive by Homeostasis Model Assessment, QUICKI=Quantitive Insulin Sensitive Check Index, SHBG=Sex Hormone Binding Globulin.*

Quantitive Insulin Check Index (QUICKI) [median (range)] had highly significant difference between the two groups. The values were as follows: in control 2.01 (1.59-2.79) and in PIH 1.90 (1.64-2.48); (p=0.002).

Both Insulin secretory capacity (HOMA%B) and Insulin sensitivity (HOMA%S) had highly significant difference between Control and PIH groups. HOMA%B and HOMA%S level in Control and in PIH groups were as follows: HOMA%B- control 101 (36.2-281.6); in PIH 166 (85-280) and HOMA%S- control 155.9 (56.3-418.6); PIH 110 (51-243), (p=<0.001).

There was also significant difference in SHBG level [(nmol/l), median (range)] between control and PIH groups. SHBG level in control was180 (103-219) vs in PIH group was 170 (10-198); (p=0.003).

## Association of PIH with various baseline parameters as explored by binary logistic regression (Table 14)

Binary logistic regression analysis with PIH as a dependent variable and parity, BMI, UPr/Cr, SHBG level and HOMA%S as independent variable, shows a highly significant association of PIH with early pregnancy BMI, SHBG, UPr/Cr and HOMA%S after adjustment of the effects of parity.

**Table 14:** Association of PIH with various baseline parameters as explored by binary logistic regression (n=142)

| Variables | β | *SE* | *p* |
|---|---|---|---|
| Parity | 0.604 | 0.510 | 0.237 |
| BMI (kg/m$^2$) | -0.178 | 0.067 | 0.008 |
| UPr/Cr | -0.259 | 0.061 | <0.001 |
| SHBG (nmol/l) | 0.022 | 0.007 | 0.002 |
| HOMA%S | 0.014 | 0.005 | 0.008 |

*β for standardized regression coefficient, PIH was taken as dependent variable whereas other variables were taken as independent variable; n = number of subjects,*

## Association of SBP and DBP at 1$^{st}$ trimester as (dependent variable) with other baseline parameters as explored by multiple regression (Table 15)

Multiple regression analysis of Systolic blood pressure ($SBP_1$) at 1$^{st}$ visit as a dependent variable (DV) with other variable, where a significant positive association was found between systolic blood pressure with parity (β=0.179; p=0.050) and negative association was found with Age (β=0.181; p=0.052).

Diastolic blood pressure ($DBP_1$) at 1st visit as a dependent variable (DV) with other variable, where a significant positive association was found between DBP with BMI (β=0.338 p=<0.001) and HDL(β=0.175 p=0.047), negative association was found with tri Glyceride(β=-0.193; p=0.026).

**Table 15:** Association of SBP and DBP at 1st trimester as (dependent variable) with other baseline parameters as explored by multiple regression (n=142)

| Parameter | SBP at 1st visit | | DBP at 1st visit | |
|---|---|---|---|---|
| | β | P | β | p |
| Age (yrs) | -0.181 | 0.052 | 0.109 | 0.212 |
| Parity | 0.179 | 0.050 | -0.024 | 0.780 |
| Gestational week | -0.013 | 0.878 | -0.026 | 0.749 |
| BMI ($kg/m^2$) | 0.122 | 0.164 | 0.338 | <0.001 |
| F glucose (mmol/L) | -0.046 | 0.591 | 0.046 | 0.569 |
| Cholesterol (mg/dl) | 0.088 | 0.360 | -0.073 | 0.421 |
| Triglyceride (mg/dl) | 0.130 | 0.156 | -0.193 | 0.026 |
| Serum creatinine (mg/dl) | -0.150 | 0.228 | 0.005 | 0.955 |
| HDL (mg/dl) | -0.106 | 0.109 | 0.175 | 0.047 |
| SHBG (nmol/L) | 0.002 | 0.437 | 0.109 | 0.216 |
| Urine Protein-Creatinine ratio (mg/mmol) | -0.073 | 0.978 | -0.005 | 0.953 |

*β for standardized regression coefficient. SHBG=Sex hormone binding globulin was taken as dependent variable whereas others were taken as independent variable; n=number of subjects, MBP=mean blood pressure; F glucose=Fasting glucose; HDL=High Density of lipoprotein, HOMA%S=Value of insulin sensitivity by Homeostasis Model Assessment.*

**Association of SBP and DBP at 2nd trimester as (dependent variable) with other baseline parameters as explored by multiple regression (Table 16)**

Multiple regression analysis of systolic blood pressure ($SBP_2$) at 2nd visit as a dependent variable (DV) with other variable, where a significant positive association was found between SBP with Parity (β=0.179;p=0.050) and negative association was found with Age (β=-0.181; p=0.052).

Diastolic blood pressure ($DBP_2$) at 2nd visit as a dependent variable (DV) with other variable, where a significant positive association was found between DBP with BMI (β=0.334;p=<0.001), UPr/Cr (β=0.173; p=0.031) and negative association was found with HDL (β=-0.210; p=0.015).

**Table 16:** Association of SBP and DBP at 2nd trimester as (dependent variable) with other baseline parameters as explored by multiple regression (n=142)

| Parameter | SBP at 2nd visit | | DBP at 2nd visit | |
|---|---|---|---|---|
| | β | P | β | p |
| Age (yrs) | -0.181 | 0.052 | 0.094 | 0.271 |
| Parity | 0.179 | 0.050 | -0.126 | 0.132 |
| Gestational week | -0.013 | 0.878 | -0.100 | 0.212 |
| BMI ($kg/m^2$) | 0.122 | 0.164 | 0.334 | <0.001 |
| F glucose (mmol/L) | -0.046 | 0.591 | -0.079 | 0.319 |
| Cholesterol (mg/dl) | 0.088 | 0.360 | 0.082 | 0.359 |
| Triglyceride (mg/dl) | 0.130 | 0.156 | 0.081 | 0.338 |
| Serum creatinine (mg/dl) | -0.106 | 0.228 | 0.069 | 0.391 |
| HDL (mg/dl) | -0.150 | 0.109 | -0.210 | 0.015 |
| SHBG (nmol/L) | -0.073 | 0.437 | 0.038 | 0.660 |
| Urine Protein-Creatinine ratio (mg/mmol) | 0.002 | 0.978 | 0.173 | 0.031 |

*β for standardized regression coefficient. SHBG=Sex hormone binding globulin, rate was taken as dependent variable whereas others were taken as independent variable; n=number of subjects, MBP=mean blood pressure; F glucose =Fasting glucose; HDL=High density of lipoprotein, HOMA%S=Value of insulin sensitivity by Homeostasis Model Assessment.*

**Association of SBP and DBP at 3rd trimester as (dependent variable) with other baseline parameters as explored by multiple regression (Table 17)**

Multiple regression analysis of Systolic blood pressure ($SBP_3$) at 3rd visit as a dependent variable (DV) with other variable, where a significant positive association was found SBP with UPr/Cr (β=0.392; p=<0.001) and negative association was found with HDL (β=-0.178; p=0.029) and SHBG (β=-0.238; p=0.004).

When the DV was DBP at 3rd visit then significantly negative association was found with SHBG (β=-0.292; p=0.001) and positive association was found with BMI (β=0.176; p=0.028) and UPr/Cr (β=0.356; p=<0.001).

**Table 17:** Association of SBP and DBP at 3rd trimester as (dependent variable) with other baseline parameters as explored by multiple regression (n=142)

| Parameter | SBP at 3rd visit | | DBP at 3rd visit | |
|---|---|---|---|---|
| | β | P | β | p |
| Age (yrs) | 0.093 | 0.246 | 0.052 | 0.533 |
| Parity | -0.070 | 0.377 | -0.093 | 0.245 |
| Gestational week | 0.103 | 0.177 | 0.036 | 0.648 |
| BMI (kg/m$^2$) | 0.129 | 0.091 | 0.176 | 0.028 |
| F Glucose (mmol/L) | -0.051 | 0.494 | 0.001 | 0.993 |
| Cholesterol (mg/dl) | 0.082 | 0.332 | 0.100 | 0.246 |
| Triglyceride (mg/dl) | 0.011 | 0.888 | 0.043 | 0.660 |
| Serum creatinine (mg/dl) | 0.129 | 0.091 | -0.036 | 0.647 |
| HDL (mg/dl) | -0.178 | 0.029 | -0.148 | 0.078 |
| SHBG (nmol/L) | -0.238 | 0.004 | -0.292 | 0.001 |
| Urine Protein-Creatinine ratio (mg/mmol) | 0.392 | <0.001 | 0.356 | <0.001 |

*β for standardized regression coefficient. SHBG=Sex hormone binding globulin, rate was taken as dependent variable whereas others were taken as independent variable; n=number of subjects, MBP=mean blood pressure; F glucose=Fasting glucose; HDL=High density of lipoprotein, HOMA%S=Value of insulin sensitivity by Homeostasis Model Assessment.*

## Sensitivity, specificity and Predictive values of SHBG, HOMA%S, UPCR, GIR and QUICK1 in the study subjects (Table 18)

Maternal serum Sex Hormone Binding Globulin (SHBG) level in the lowest 50th percentile at earlier weeks of gestation can predict PIH in 39% with NPV 80%, sensitivity 63% and specificity 80%. SHBG level in the lowest 25th percentile can predict PIH in 34% with NPV 79%, sensitivity 34% and specificity 79%.

**Table 18:** Predictive values of SHBG, HOMA%S, UPCR, GIR and QUICK1 in the study subjects (n=142)

| Variable | Percentile | Cut-off Value | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|
| **SHBG level** | 20th | 133.0 | 26% | 81% | 36% | 73% |
| | 25th | 149.0 | 34% | 79% | 34% | 79% |
| | 50th | 180.0 | 63% | 80% | 39% | 80% |
| **HOMA%S** | 50th | 139.0 | 24% | 35% | 13% | 55% |
| | 75th | 173.37 | 7% | 68% | 8% | 64% |
| **UPr/Cr** | 50th | 5.65 | 85% | 59% | 46% | 90% |
| | 65th | 6.79 | 65% | 78% | 55% | 84% |
| | 75th | 9.82 | 46% | 85% | 55% | 79% |
| **GIR** | 25th | 0.64 | 58% | 28% | 25% | 63% |
| | 50th | 0.76 | 34% | 44% | 2% | 62% |
| | 75th | 0.92 | 12% | 71% | 14% | 66% |
| **QUICK1** | 25th | 1.86 | 69% | 19% | 26% | 62% |
| | 50th | 1.96 | 36% | 39% | 19% | 60% |
| | 75th | 2.11 | 17% | 66% | 17% | 66% |

*McNamara test was done as predictivity; PPV,=Positive predictive value; NPV= Negative predictive Value; BMI=Body mass Index; SHBG=Sex hormone binding globulin; HOMA %S=Insulin sensitive by Homeostasis Model Assessment, UPr/Cr=Urine protein creatinine ratio; GIR=Glucose insulin ratio; QUICK1=Quantitive insulin sensitivity check index.*

SHBG level in the lowest 20th percentile can predict PIH in 36% with NPV 73%, sensitivity 26% and specificity 81%. SHBG level below the cut-off value (median value) can predict PIH in 37% with NPV 76%, sensitivity 48% and specificity 66%.

Urine Protein-Creatinine ratio (UPr/Cr) in the highest 50th percentile at earlier weeks of gestation can predict pregnancy induced hypertension in 46% with NPV 90%, sensitivity 85% and specificity 59%. UPr/Cr in the highest 65th

percentile at earlier weeks of gestation can predict pregnancy induced hypertension in 55% with NPV 84%, sensitivity 65% and specificity 78%. UPr/Cr in the highest 75$^{th}$ percentile can predict PIH in 55% with NPV 79%, sensitivity 46% and specificity 85%.

HOMA%S in the highest 50$^{th}$ percentile at earlier weeks of gestation can predict pregnancy induced hypertension in 13% with NPV 55%, sensitivity 24% and specificity 35%. And the highest 75$^{th}$ percentile at earlier weeks of gestation can predict pregnancy induced hypertension in 8% with NPV 64%, sensitivity 7% and specificity 68%.

Glucose-Insulln ratio in the highest 25$^{th}$ percentile can predict PIH in 25% with NPV 63%, sensitivity 58% and specificity 28%. The highest 50$^{th}$ percentile at earlier weeks of gestation can predict pregnancy induced hypertension in 2% with NPV 62%, sensitivity 34% and specificity 44%. And the highest 75$^{th}$ percentile at earlier weeks of gestation can predict pregnancy induced hypertension in 14% with NPV 66%, sensitivity 12% and specificity 71%.

QUICKI in the highest 25$^{th}$ percentile can predict PIH in 26% with NPV 62%, sensitivity 69% and specificity 19%. The highest 50$^{th}$ percentile at earlier weeks of gestation can predict pregnancy induced hypertension in 19% with NPV 60%, sensitivity 36% and specificity 39%. And the highest 75$^{th}$ percentile at earlier weeks of gestation can predict pregnancy induced hypertension in 17% with NPV 66%, sensitivity 17% and specificity 66%.

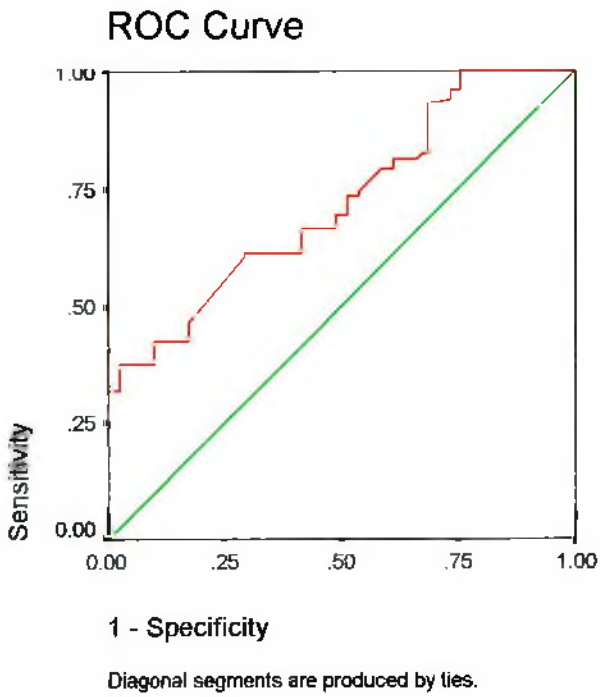


**Figure 2:** ROC curve analysis for SHBG level as a predictor of significant in PIH.

ROC curve was constructed on the basis of several cut-off point of SHBG. Area under the ROC curve is 0.719 and optimal value that maximized sensitivity and specificity for a predictor.

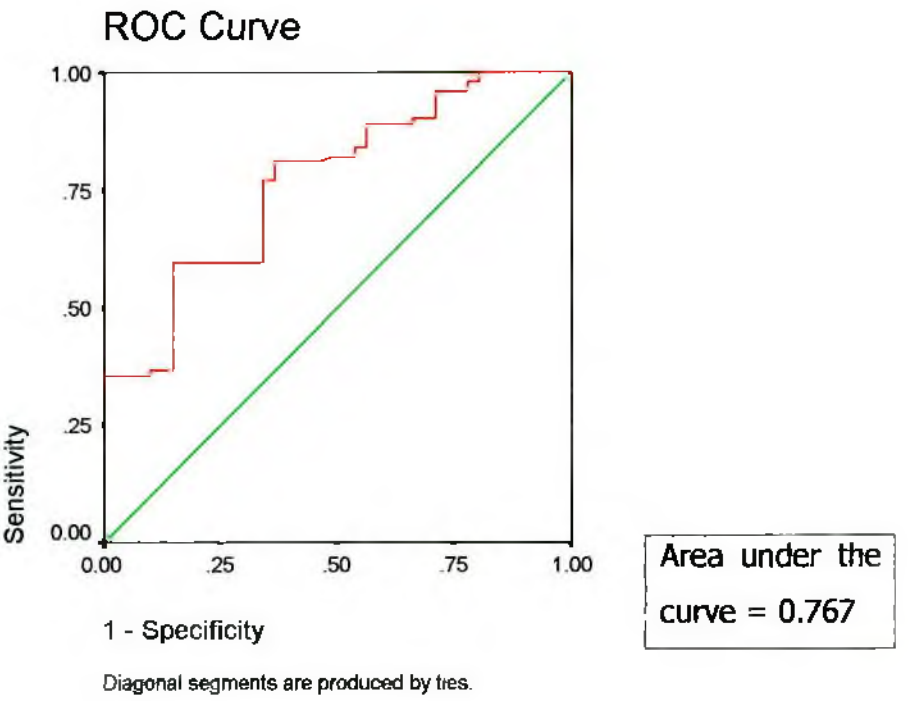


**Figure 3:** ROC curve analysis for UPr/Cr as a predictor of significant in proteinurea.

ROC curve was constructed on the basis of several cut-off point of UPr/Cr. Area under the ROC curve is 0.767 and optimal value that maximized sensitivity and specificity for a predictor.

## Association of PE with various baseline parameters as explored by binary logistic regression (Table 19)

Binary logistic regression analysis with PIH as a dependent variable and parity, BMI, Upr/Cr, SHBG level and HOMA%S as independent variable, shows a highly significant association of PIH with early pregnancy SHBG after adjustment of the effects of parity, BMI, Upr/Cr, SHBG level and HOMA%S.

**Table 19:** Association of PE with various baseline parameters as explored by binary logistic regression

| Variables | β | *SE.* | *p* |
|---|---|---|---|
| Parity | 0.012 | 1.198 | 0.992 |
| BMI (kg/m$^2$) | -0.002 | 0.132 | 0.990 |
| UPr/Cr | 0.048 | 0.119 | 0.684 |
| SHBG (nmol/l) | -0.076 | 0.023 | 0.001 |
| HOMA%S | -0.007 | 0.012 | 0.545 |

*β for standardized regression coefficient. PE was taken as dependent variable whereas other variables were taken as independent variable; n = number of subjects,*

## Association of GH with various baseline parameters as explored by binary logistic regression (Table 20)

Binary logistic regression analysis with PIH as a dependent variable and parity, BMI, Upr/Cr, SHBG level and HOMA%S as independent variable, shows a highly significant association of PIH with early pregnancy BMI, Upr/Cr and HOMA%S. after adjustment of the effects of parity and SHBG.

**Table 20:** Association of GH with various baseline parameters as explored by binary logistic regression

| Variables | β | *SE.* | *p* |
|---|---|---|---|
| Parity | -0.660 | 0.504 | 0.190 |
| BMI (kg/m$^2$) | 0.182 | 0.063 | 0.004 |
| UPr/Cr | 0.212 | 0.054 | <0.001 |
| SHBG (nmol/l) | 0.004 | 0.006 | 0.525 |
| HOMA%S | -0.014 | 0.005 | 0.006 |

*β for standardized regression coefficient. GH was taken as dependent variable whereas other variables were taken as independent variable; n = number of subjects,*

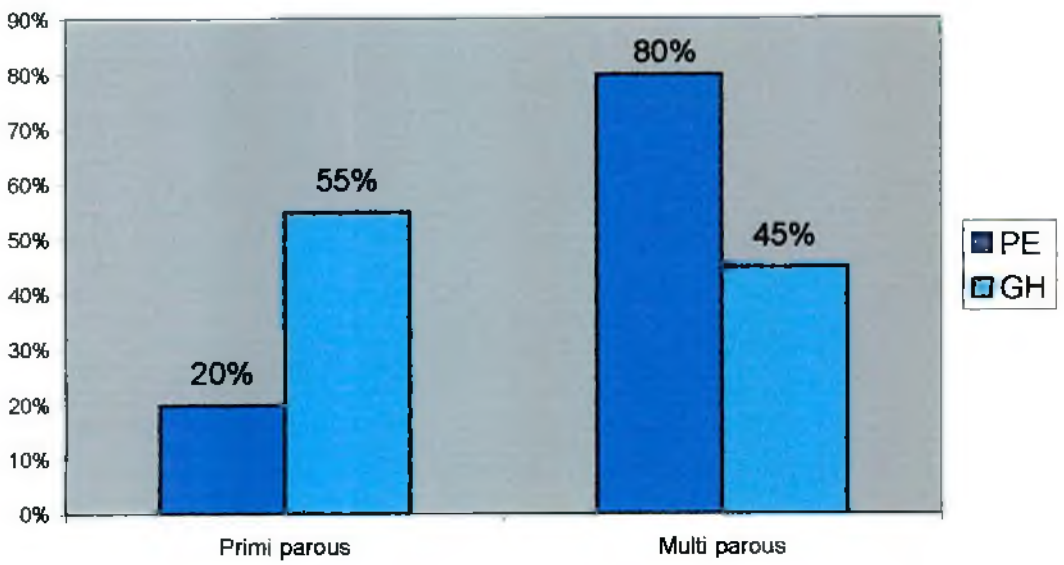


**Figure 4:** Proportiopn of primiprimipara and multipara in PE and GH cases.

**Proportiopn of primiprimipara and multipara in PE and GH cases (Figure 4)**

PE occurred mostly in nulliparous cases but there was no significant difference between nullipara and multipara regarding the occurrence of GH, rather there was a higher proportion of GH in primigravida.

**Table 21**: Relation of SHBG with various parameters of the study subjects (n=142)

| Parameter | r | p |
|---|---|---|
| Age (Yrs) | -0.143 | 0.090 |
| BMI (kg/m$^2$) | 0.007 | 0.938 |
| Parity | -0.286 | 0.001 |
| Gestational Wk | 0.045 | 0.593 |
| SBP (mm Hg) | 0.216 | 0.010 |
| DBP (mm Hg) | 0.112 | 0.183 |
| MBP (mm Hg) | 0.177 | 0.035 |
| SBP2 (mm Hg) | 0.063 | 0.455 |
| DBP2 (mm Hg) | 0.093 | 0.272 |
| MBP2 (mm Hg) | 0.084 | 0.320 |
| SBP3 (mm Hg) | -0.210 | 0.012 |
| DBP3 (mm Hg) | -0.248 | 0.003 |
| MBP3 (mm Hg) | -0.238 | 0.004 |
| F glucose (mmol/l) | 0.045 | 0.594 |
| Triglyceride (mg/dl) | -0.098 | 0.246 |
| Cholesterol (mg/dl) | 0.062 | 0.464 |
| HDL (mg/dl) | -0.188 | 0.025 |
| LDL (mg/dl) | 0.210 | 0.012 |
| Creatinine (mg/dl) | -0.161 | 0.056 |
| Urine Protein-Creatinine ratio (mg/mmol) | -0.203 | 0.015 |
| Insulin | 0.191 | 0.023 |
| Glucose-Insulin ratio | 0.147 | 0.081 |
| QUICKI | 0.136 | 0.105 |
| HOMA%B | -0.054 | 0.527 |
| HOMA%S | -0.124 | 0.141 |

*Pearson's parametric correlation coefficient's and Spearman's nonparametric correlation coefficient's test was done as a test of significance as appropriate.*

**Relation of SHBG with various parameters of the study subjects (Table 21)**

Significant positive correlation was found with SBP (r=0.216, p=0.010), MBP (r=0.177, p=0.035) at first visit, LDL(r=0.210, p=0.012) and fasting insulin (r=0.191, p=0.023), and significant negative correlation was found with $SBP_3$(r=-0.210, p=0.012), HDL (r=-0.188, p=0.025), creatinine (r=-0.161, p=0.054) and Upr/cr (r=-0.203, p=0.015).

A highly significant positive correlation of SHBG was found with parity(r=-0.286, p=0.001) DBP (r=-0.248, p=0.003) and MBP (r=-0.238, p=0.004) at the 3rd visit.

**Table 22:** Association of SHBG as (dependent variable) with other baseline parameters as explored by multiple regression (n=142)

| Parameter | β | p |
|---|---|---|
| Age (Yrs) | -0.043 | 0.623 |
| BMI ($kg/m^2$) | -0.057 | 0.524 |
| Parity | 0.225 | 0.007 |
| Gestational Wk | 0.052 | 0.515 |
| MBP (mm Hg) | 0.186 | 0.039 |
| Triglyceride (mg/dl) | -0.115 | 0.188 |
| Cholesterol (mg/dl) | 0.222 | 0.012 |
| HDL (mg/dl) | -0.245 | 0.005 |
| UPr/Cr | -0.195 | 0.013 |
| HOMA%S | 0.112 | 0.163 |

*β for standardized regression coefficient. SHBG=Sex hormone binding globulin, rate was taken as dependent variable whereas others were taken as independent variable; n=number of subjects, MBP=mean blood pressure; HDL=High density of lipoprotein, HOMA%S=Value of insulin sensitivity by Homeostasis model assessment.*

**Association of SHBG as (dependent variable) with other baseline parameters as explored by multiple regression (Table 22)**

Multiple regression analysis of SHBG was done as a dependent variable (DV) with other variable, where a significant positive association found between parity (β=0.225;p=0.007), mean blood pressure (β=0.186;p=0.039), cholesterol (β=0.222;p=0.012), HDL (β=-0.245;p=0.00) and UPr/Cr (β=-0.195;p=0.013).

**Relation of UPr/Cr with various parameters of the study subjects (Table 23)** (n=142)

A significant negative correlation was found with Fasting Glucose (r=-0.193, p=0.021). A highly significant positive correlation was found with SBP (r=0.340, p=<0.001), DBP (r=0.341, p=<0.001), MBP (r=0.353, p=<0.001) at 3$^{rd}$ visit and HOMA%B (r=0.250, p=0.003).

**Table 23**: Relation of UPr/Cr with various parameters of the study subjects (n=142)

| Parameter | r | p |
|---|---|---|
| Age (Yrs) | 0.019 | 0.819 |
| BMI (kg/m$^2$) | -0.102 | 0.228 |
| Parity | -0.039 | 0.688 |
| Gestational Wk | 0.061 | 0.468 |
| SBP (mm Hg) | -0.114 | 0.273 |
| DBP (mm Hg) | -0.115 | 0.172 |
| MBP (mm Hg) | -0.098 | 0.246 |
| SBP2 (mm Hg) | -0.013 | 0.874 |
| DBP2 (mm Hg) | 0.062 | 0.461 |
| MBP2 (mm Hg) | 0.049 | 0.564 |
| SBP3 (mm Hg) | 0.340 | <0.001 |
| DBP3 (mm Hg) | 0.341 | <0.001 |
| MBP3 (mm Hg) | 0.353 | <0.001 |
| F Glu (mmol/L) | -0.193 | 0.021 |
| Triglyceride (mg/dl) | 0.012 | 0.884 |
| Cholesterol (mg/dl) | 0.015 | 0.857 |
| HDL (mg/dl) | 0.033 | 0.696 |
| LDL (mg/dl) | -0.073 | 0.386 |
| Creatinine (mg/dl) | -0.062 | 0.461 |
| SHBG (nmol/L) | -0.129 | 0.461 |
| Insulin | -0.083 | 0.329 |
| Glucose/Insulin ratio | 0.001 | 0.993 |
| QUICKI | 0.034 | 0.689 |
| HOMA%B | 0.250 | 0.003 |
| HOMA%S | -0.007 | 0.934 |

*Pearson's parametric correlation coefficient's and Spearman's nonparametric correlation coefficient's test was done as a test of significance as a appropriate.*

**Association of UPr/Cr as (dependent variable) with other baseline parameters as explored by multiple regression (Table 24)**

Multiple regression analysis of UPr/Cr as a dependent variable (DV) with other variable, marginally significant association was found only with SHBG (β=-0.194;p=0.050).

**Table 24:** Association of UPr/Cr as (dependent variable) with other baseline parameters as explored by multiple regression (n=142)

| Parameter | β | p |
|---|---|---|
| Age (yrs) | 0.097 | 0.327 |
| BMI (kg/m$^2$) | -0.076 | 0.441 |
| Parity | -0.081 | 0.396 |
| Gestational week | 0.005 | 0.955 |
| SBP (mm Hg) | -0.018 | 0.843 |
| DBP (mm Hg) | -0.061 | 0.776 |
| MBP (mm Hg) | 0.071 | 0.751 |
| F glucose (mmol/L) | -0.141 | 0.111 |
| Triglyceride (mg/dl) | 0.055 | 0.603 |
| Cholesterol (mg/dl) | 0.326 | 0.158 |
| HDL (mg/dl) | -0.192 | 0.091 |
| LDL (mg/dl) | -0.326 | 0.117 |
| SHBG level (nmol/l) | -0.194 | 0.050 |
| HOMA%S | -0.026 | 0.772 |

*β for standardized regression coefficient. SHBG=Sex hormone binding globulin, rate was taken as dependent variable whereas others were taken as independent variable; n=number of subjects, MBP=mean blood pressure; F glucose=Fasting glucose; HDL=High Density lipoprotein, HOMA%S=Value of Insulin Sensitivity by Homeostasis Model Assessment.*

# Chapter 5

# DISCUSSION

# 5. DISCUSSION

## *Prevalence of PIH*

Although PIH is known to be a relatively common complication of pregnancy in developing countries, no epidemiological data in Bangladeshi population has yet been reported. Lack of proper management records precludes the conduction of even retrospective analysis. The present sample consisted of pregnant subjects collected from different public hospitals representing various socioeconomic conditions. The subjects originated mainly from urban background. The total number of pregnant women was 404; with 95% confidence interval and 0.025 standard error the study gives a power of more than 80% to calculate the prevalence of PIH. This power is standard minimum for statistical reasoning in medicine and thus the present data can form the basis of large scale epidemiological studies in Bangladeshi population.

Analysis of the present data show a prevalence of 10.14% PIH among pregnant women in hospital settings of Dhaka city. The findings are consistent with those observed by Wolf et al (2002) and Solomon et al (2003) where they found that PIH complicates 5-10% of pregnancies respectively in the United States. Cunninghm et al (2007) have shown a 10% incidence of PIH among pregnant women worldwide. In a report by National High Blood Pressure Education Programme (2000) it was shown that PIH occurs in 8% of pregnancies. The data from a maternity based retrospective study in Iceland showed the incidence of PIH to be much higher (17.4%) (Gunnlaugsson et al, 1989); on the other hand, a hospital based survey reported by Huang (2001) has shown a much lower incidence in Shanghai (5.57%). These differences can reasonably be explained by racial and environmental variation as well as level of health awareness and care. The present population is urban and hospital based, so the actual prevalence in

the community, particularly in the rural areas, can be expected to be substantially higher.

When PIH was divided into its two subtypes (PE and GH) the statistical power of analysis became lower, even then a preliminary estimate on the prevalence could be made. The frequency of PE was found to be 2.47% and that of GH was found to be 7.67%. Although this data needs further validation, it compares favorably with data from population-based studies by Gibson et al (2007) who reported that approximately 1-2% by of pregnancies are complicated by PE and 5-6% by GH. The reported incidence of GH (15%) and PE (7%) in USA (www.wrong diagnosis.com) is higher than our findings. Moodley et al (1999) reported that PE affects 4.4% of all deliveries and may be as high as 18% in some settings in Africa, which was higher than the present findings. From a hospital based study Krishna et al (1994) has shown a higher incidence of PE (8-10%) in India. As stated previously the present data is on urban hospital based population and thus the prevalence of PE may be substantially higher in rural communities which will be more in conformity with the prevalence in Africa and India.

### *Factors associated with the Prevalence of PIH*

The association of PIH with age, parity, rural-urban distribution and socioeconomic condition was first analyzed by a case-control comparison. No parameter, except parity, was found to be different between PIH and non–PIH groups. Multiparous women are reported to have higher incidence of PIH (MacGillivray,1983) and it is in conformity with the present finding. Separate analysis of PE shows the same trend, but such difference is not seen in GH.

Robson (1999) reported that PIH occurs in about 16-24% of first pregnancies and 12-15% of subsequent pregnancies, which is similar with our findings. Robson also observed that PE complicates 3-5% first pregnancies and 1% of subsequent pregnancies. MacGillivray et al (1983) and Gunnlaugsson et al (1989) have

reported a higher prevalence of PE in parous women, but the present data contradict the findings of Mounier-Vehier et al (1999) and Wolf et al (2002) where it was found that PIH is more common among primigravida patients than multigravida ones.

The association of PIH with baseline parameters were further explored by logistic regression analysis with PIH as the dependent variable (Table 5). In parallel with the result of the significance test for group difference only parity turns out to be significantly associated with PIH.

### *Baseline characteristics of PIH cases in relation to Control*

Under a nested case-control design 101 non-PIH cases with matched age and gestational week were selected as Control and their baseline clinical and anthropometric characteristics were compared. In the absence of the confounding effects of age and gestational week a positive history of hypertension (but not PE) in earlier pregnancy was found in 71% of PIH cases in contrast to only 32% in Control. Persson et al (1998) reported a higher incidence of PIH in cases with previous history of GH, which conforms to the present finding. None of the baseline blood pressure differed between the two groups, thus this parameter does not yet give any indication about the future development of hypertension in these patients. Baseline biochemical parameters also did not differ between the two groups.

### *Association of nutritional factors with PIH*

Calcium deficiency has been implicated as a possible cause of GH (Hamet et al (1995), Bucher et al, 1996; Leela et al, 1991) and PE (Belizan and Villar, 1980; Ramos et al, 2006;Villar et al, 2003)). Loverro et al, 1996 and Oostenburg et al (1998) reported that antioxidant levels in the blood of women with GH appear to be reduced but the findings of Gratacos et al (1998) was different. Belizan et al (1983), Banerjee, Chambers & Campbell (2006) and Rumiris et al (2006) have

suggested the association of vitamin C and E with PE. The intake of major nutritional factors in PIH and Control cases were assessed by recall method. None of the factors differed between the two groups except a marginally lower intake of iron and vitamin $B_2$. Regarding iron (Scholl, 1994) and riboflavin (Zamzam et al, 2007) intake the present findings are similar with the findings of Scholl (1994) and Zamzam (2007) respectively. Zamzam et al (2007) found an association of lower intake of calcium, zinc, riboflavin and protein intake with PIH, but in the present work, none of these factors in early pregnancy was associated with PIH in late pregnancy.

### *Association PIH with early endothelial dysfunction*

Urinary protein-creatinine ratio is now well recognized as a marker of microvascular damage arising from endothelial dysfunction. It has been reported to be raised in essential hypertension (Wendy et al, 2000) as well as in PE (Ragip et al, 2004) and GH or both (Miranda et al, 2007; Villar et al, 1989; Boler et al, 1987; Jaschevatzky et al, 1990, Kaplan et al, 1989). In the present study this marker at baseline was already almost twice in PIH compared to Control ($p=<0.001$). Although association of PIH with raised UPr/Cr has been shown before in established cases, the baseline rise of this parameter in early pregnancy of PIH subjects has not yet been reported. The present finding strengthens the possibility of involvement of endothelial damage as a causal factor in PIH.

### *Association of PIH with early insulin resistance in pregnancy*

Several indices of insulin resistance were explored in this study for their association with PIH. Blood glucose, plasma insulin and glucose/insulin ratio are used as surrogate markers of insulin resistance (McAuley et al, 2001; Makiko et al, 2007). Glucose-Insulin ratio was significantly lower in the PIH group indicating

insulin resistance in early pregnancy of these cases (Table 13) (Anneli et al, 2004; Olup et al, 2005). Sermer et al (1995) and Joffe et al (1998) also reported a similar data in GH cases. Early hyperinsulinemia in PIH was further evident with significantly higher insulin secretory capacity in PIH as evident by HOMA%B values. QUICKI also demonstrated early insulin resistance in PIH. Further evidence of insulin insensitivity came from HOMA%S and SHBG values both of which showed significantly lower level in PIH as compared to Control. Some studies have shown decreased HOMA%S levels in women with established PIH (Kocyigit etal, 2004 and Weisz et al, 2005). Jesus et al (2007) have shown significant association of lower HOMA%S in early pregnancy and subsequent development of PIH. Association of lower HOMA%S in diagnosed GH cases has been shown by Romero et al (2004), but not in PE cases. The same investigators, Romero et al (2003), did not find an association between PIH and IR as assessed by HOMA in the third trimester. Similarly, fall of SHBG in these cases has been reported by Wolf et al (2002). But report on the association of reduced SHBG at early stages of pregnancy with PIH at later stages have not been reported before.

### *Association of PIH with endothelial dysfunction and insulin resistance after adjustment of effects of the confounders*

The pathophysiology of PIH is quite complex involving interaction of multiple factors both from genetic and lifestyle origin. Although the present study was designed with insulin resistance as the primary outcome variable, it turned out from analysis that early pregnancy endothelial dysfunction (a covariate of insulin resistance) has an equally strong association with PIH. Accordingly, the present data required further in-depth analysis to explore these associations arising from the case-control comparisons. Pulling the data into a single class of pregnant women the association of two or more variables could be analyzed by bivariate as well as multivariate statistics. On Spearman's correlation analysis UPr/Cr was not found to have any correlation with any of the parameters; however, SHBG gave a

significant correlation with systolic blood pressure in the early weeks. The risk for developing PIH in relation to SHBG was further analyzed and with 50$^{th}$ percentile of SHBG (170 nmol/l) as a cut-off point the PIH cases showed 2.78 times higher Odds (p=<0.006) of having lower SHBG as compared to control. Similar evidence of insulin resistance in early pregnancy has been shown by Wolf et al (2002) and Marshal et al (2007), but their patients had higher BMI which itself is a strong confounder of insulin resistance. The demonstration of this abnormality in our lean pregnant patients provides a stronger evidence of association of insulin resistance with PIH.

The evidence is further substantiated by the results of the logistic regression where PIH was taken as the dependent variable and various indices of insulin resistance, along with their covariates, were taken as independent variables (Table 14). Among the indices HOMA%S and SHBG showed strong association with PIH even when the effects of other covariates were adjusted. Such association with HOMA%S, under a logistic regression model, has been previously claimed by Wolf et al (2002), but the subjects were of higher BMI and the strength of the association was weaker. No work with SHBG has yet been reported on this issue. The possible causal role of insulin resistance in PIH is substantiated by the present findings. A similar association of PIH with UPr/Cr, even after adjustment of the effects of other confounders, was found in logistic regression analysis (Table 24). This provides strong evidence of a possible causal role of endothelial damage in PIH.

### *UPr/Cr, HOMA%S and SHBG as predictors of PIH*

Demonstration of the association of PIH with early pregnancy values of UPr/Cr as well as indices of insulin resistance do not necessarily ensure the predictive value of these parameters in the development of the disorder in later stages of pregnancy. Thus, extensive analysis was done in this regard with various parameters at different cut-off points and ROC curves were constructed with

SHBG and UPr/Cr which appeared to be the probable predictive markers (Figure 3).

From the ROC curve the most appropriate cut-off value of SHBG was selected. The 50$^{th}$ percentile of cut-off value (180 nmol/l) was found to be optimum where the sensitivity of SHBG is 63% and PPV 39%. The corresponding maximum values for HOMA%S is 24% and 13%, which show that SHBG is a much better predictive marker of PIH than HOMA%S. Although sensitivity and PPV of SHBG is not very high, but it is quite relevant clinically when looked from the standpoint of the relatively low frequency of the disease.

The early pregnancy uterine Doppler reported 20% sensitivity for PIH (James et al, 2006). Compared to Doppler technique SHBG measurement is technically much simpler and economically more cost-effective. The blood sample collection for SHBG does not involve fasting and this parameter is not susceptible to diurnal variation. The gold standard test for insulin sensitivity (clamp technique) is not feasible for pregnant subjects in a clinical setting and SHBG gives a high degree of correlation with the data from clamp technique. Thus, measurement of this globulin (which can be easily practiced in pregnancy) seems to be a useful practice in early pregnancy for predicting the late development of PIH.

Since failure to diagnose 37% of cases of PIH does not lead to any extra instructions which can lead to increased risk to the pregnant women, the 63% sensitivity with such a laboratory test can help in the measurement of a large number of potential PIH cases. The usefulness of the test is further exemplified by its high specificity (80%) and NPV (80%) as the chance of false negativity is quite low and thus most of the patients are not given an extra burden and psychological stress regarding their management.

A similar argument can be put forward for UPr/Cr, which is even easier and cheaper then SHBG, and can be performed ever in clinical laboratories at rural settings. In fact, UPr/Cr turned out to be an even better test than SHBG in predicting PIH. The optimum cut-off value (6.79) in case of UPr/Cr was found to

Lie et al 65$^{th}$ percentile which gives a sensitivity of 65%, PPV 55%, Specificity 78% and NPV 84%.

### *Are GH and PE different entities*

It is still uncertain whether, pathophysiologicaiiy, GH and PE are separate disorders or PE is just a form of GH progressing to protelnurea (Barton et al, 2001). In the present study the issue was addressed by comparing the parameters in GH and PE groups, which showed significant, difference only in SHBG (p=<0.001) and HDL (p=0.015) on group comparison analysis. A major difference was found in the logistic regression: the urinary-protein creatinine ratio showed highly significant association with GH (but not with PE). On the other hand, SHBG shows highly significant association with PE, but not with GH. Macrovascular damage is claimed to have an etiological role in essential hypertension and finding of elevated Alb-Cr ratio is thought to be a predictor of essential hypertension (Bolero, Bella & Bleacher, 1987; Robert et al, 1997). It is interesting to see that UPr/Cr in the present study has association only with GH. The pathophysiology of PE is complex and insulin resistance is thought to play a key role in this disorder. Large-scale prospective studies can only address these issues more conclusively.

### *Determinants of SHBG and UPr/Cr in PIH*

Univariate analysis showed that SHBG has a negative association with only parity and it is positively associated with diastolic blood pressure ($DBP_2$) and mean blood pressure ($MBP_2$) in second trimester (p=<0.001).

On logistic regression analysis SHBG was found to be significantly associated with parity, mean blood pressure, dyslipidemia and endothelial damage. All these are known risk factors for insulin resistance.

The probable determinants of endothelial damage in PIH were also explored by testing the association of UPr/Cr (dependent variable) with age, BMI, parity, blood pressure, blood glucose, lipid profile, insulin and SHBG in multiple regression analysis. The association was not found to be significant except with SHBG. The association between UPr/Cr and SHBG, after adjusting the other confounders, is an interesting one as insulin resistance is often implicated in the pathophysiology of endothelial dysfunction in disorders of metabolic syndrome (Rowe et al, 1981; Reaven, Lithell and Landsberg, 1996; Defronzo et al, 1975; Doria et al, 1991; Gibbons and Dzau, 1994).

## *Conclusions*

The present data lead to the following conclusions:

a. In Bangladesh, the prevalence of PIH in urban hospital based pregnant population is 10.14% with almost 3 times higher proportion of preeclampsia than gestational hypertension;

b. Multiparity is a major risk factor for PIH;

c. Insulin resistance and endothelial dysfunction seem to have causal association with PIH;

d. Early pregnancy SHBG and UPr/Cr can be clinically useful predictors of PIH at late pregnancy. Of these two options, UPr/Cr has a little better sensitivity and specificity and this can be preferred as it is technically much simpler (implementable even in rural settings) and cost-wise more economic.

# Chapter 6

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# 6. REFERENCE

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# Chapter 7

# APPENDICES

## Appendix-I

### "সম্মতি পত্র"

আমি ডাঃ রোমেনা হেলাল কর্তৃক অবগত হই যে, গর্ভাবস্থায় বিভিন্ন জটিলতার উপর বারডেম হাসপাতালে একটি গবেষনা কাজ চলিতেছে। তিনি এই গবেষনা কাজের বিভিন্ন দিক আমাকে বুঝিয়ে বলেন।

আমি এই মর্মে বুঝতে পেরেছি যে, গর্ভাবস্থায় বিভিন্ন জটিলতার চিকিৎসা কাজে সুফল বয়ে আনার জন্য এই গবেষণা কাজটি পরিচালিত হচ্ছে। আমি এই গবেষণা কাজটির উদ্দেশ্য, ধরণ এবং সময়কাল সম্পর্কে পরিষ্কার ধারণা পেয়েছি। আমি এটাও বুঝেছি যে, এই গবেষণা কাজে সংগৃহীত আমার ব্যক্তিগত পরিচয় এবং অন্যান্য সংবেদনশীল সামাজিক তথ্যাবলী সম্পূর্ণ গোপন রাখা হবে। আমাকে বলা হয় যে, ৮-১০ ঘণ্টা খালি পেটে থাকার পর সকাল বেলা আমাকে ৮ সিসি রক্ত দিতে হবে। ৭৫গ্রাম গ্লুকোজ খাওয়ার ২ ঘণ্টা পর পুনরায় ২ সিসি রক্ত দিতে হবে। এই সাক্ষাৎকারের জন্য নির্ধারিত সময় ৪০ মিনিট।

আমি এটাও বুঝেছি যে, এই গবেষণা পদ্ধতিগত সংক্রান্ত যে কোন প্রশ্ন করার সুযোগ আমাকে দেওয়া হবে এবং পরবর্তীতে কোন প্রশ্ন থাকলে গবেষণা কারীর সাথে যোগাযোগ করা যাবে। আমি বুঝেছি যে, কোন প্রকার মাশুল ও কারণ দর্শানো ব্যতিরেকে যে কোন সময়ে এই গবেষণার কার্যক্রম ত্যাগ করতে অথবা সম্মতি উঠিয়ে নিতে আমার অধিকার থাকবে।

আমি স্বেচ্ছায় এবং স্বজ্ঞানে এ গবেষণা কাজে অংশগ্রহণ করতে রাজি আছি।

অংশগ্রহণকারীর স্বাক্ষর / টিপসই (নিরক্ষর হলে)

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নাম ঃ

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পিতা / স্বামীর নাম ঃ

ঠিকানা ঃ

তারিখ ঃ

Rose Helal

গবেষণাকারীর স্বাক্ষর

# Appendix- II

## DATA COLLECTION SHEET

**ID:** ☐ **Date:**

**Name:** **Institution:**

**Age:**

**Husband's name:**

**Address:** **a) Present:**

Telephone No:

**b) Permanent Address:**

Contact person address:

Telephone No:

**Education:** ☐ Masters ☐ Graduate ☐ HSC ☐ SSC ☐ Primary

**Occupation:** ☐ Housewife ☐ Service ☐ Students

**Social status:** ☐ Urban ☐ Semi Urban ☐ Rural

**Gestational age at entry in the study:**

**Antenatal care:** 1st trimester /week- ☐
2nd trimester / week- ☐
3rd trimester / week- ☐

**Family history of hypertension:** i) Present | Father | Mother | Brother | Sister |
ii) Absent

**Family history of DM:** i) Present | Father | Mother | Brother | Sister |
ii) Absent

**Menstrual history:** MP/MC ☐ L.M.P ☐ E.D.D ☐

**Obstetrical history:** Para ☐ Gravida ☐ ALC ☐

**Previous obstetric history:** i) Present | PE | GDM | Eclampsia |
ii) Absent

**Medical history:** i) Present | DM | HTN | Thyroid disorder |
ii) Absent

**Present drug history (Including vitamins and minerals):**

| Trade name | Dose | mg | Generic name |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

**Physical/Clinical Examination:**

| Examination | 1st trimester (1 m – 3 m) | 2nd trimester (4 m – 6 m) | | | 3rd trimester (7 m – 9 m) | | |
|---|---|---|---|---|---|---|---|
| | weeks (1 m – 3 m) | weeks (13 – 16) | weeks (17 – 20) | weeks (21 – 24) | weeks (25 -28) | weeks (29 –32) | weeks (33 - ) |
| Height | | | | | | | |
| Weight | | | | | | | |
| BP | | | | | | | |
| Anemia | | | | | | | |
| Edema | | | | | | | |
| Ht of Uterus | | | | | | | |
| Urinary protein (by strip) | | | | | | | |

**Biochemical Investigations:**

- Serum F Glu (mmol/L):
- 2hr after 75gm Glu (mmol/L):
- Serum TG (mg/dL):
- Serum T-chol (mg/dL):
- Serum HDL (mg/dl):
- Serum LDL (mg/dL):
- Serum Creatinine (mg/dL):
- Serum Insulin (ngm/mL):
- Serum Sex Hormone Binding Globulin (nmol/L):
- Urine Total Protein (g/L):
- Urine Total Creatinine (mg/dL):

## Appendix- III

**Estimation of serum blood glucose** (Randox laboratories, UK).

Serum glucose was estimated by enzymatic colorimetric (GOD-PAP) method

***Principle*** *(Barham, Trinder, 1972)*

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red violet quinoneimine dye as indicator.

$$\text{Glucose} + H_2O \xrightarrow[\text{Oxidase}]{\text{Glucose}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

*Reagents*

| *Contents* | *Initial concentration of soiution* |
|---|---|
| Buffer | |
| Phosphate Buffer | 0.1 mol/l, pH 7.0 |
| Phenol | 11 mol/l |
| GOD-PAP Reagent | |
| 4-aminophenazone | 0.77 mmol/l |
| Glucose oxidase | ≥1.5 kU/l |
| Peroxidase | ≥1.5 kU/l |
| Standard | |
| Glucose | 5.55 mmol/l (100 mg/dl) |

### *Additional Reagent*

Uranyl Acetate 0.16% cat NO DP 647 2x 500 ml

### *Materials required*

Microcentrifuge tube

Micropipettes and pipettes with disposable tips

AUTOLAB (Analyzer Medical system, Rome, Italy)

### *Procedure*

Procedure for glucose GOD-PAP assay without deproteinization. The instrument was calibrated before estimation.

Serum and reagent were taken in specific cup. They were arranged serially into the Auto lab Analyzer (Analyzer Medical system, Rome, Italy). The Auto lab was programmed for the estimation of glucose and allowed to run with following procedure:

5 μl sample and 500 μl reagent were mixed and incubated at 370 C for 10 minutes. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

### *Calculation of result*

Optical densities or absorbances were fed into a computer and calculation was done using the software program. Values for the unknown samples were calculated by extrapolating the absorbance for the standard using following formula.

$$\text{Glucose concentration (mmol/l)} = \frac{A_{Sample}}{A_{Standard}} \times 5.55$$

## Appendix- IV

### Estimation of serum Total Cholesterol

Total cholesterol was measured by enzymatic endpoint method (cholesterol Oxidase/Peroxidase) method in auto alyzer (Analyzer Medical System, Rome, Italy) using reagent of Randox laboratories, UK (Trinder. 1988)

***Principle***

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantiphyrine in the presence of phenol and peroxidase.

$$\text{Cholesterol ester} + H_2O \xrightarrow[\text{Esterase}]{\text{Cholesterol}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Oxidase}]{\text{Cholesterol}} \text{Cholestene-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{phenol} + \text{4-aminoantiphyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + H_2O$$

***Reagent composition***

| Contents | Initial Concentration of Solution |
|---|---|
| ***Reagent*** | |
| 4-Aminoantipyrine | 0.30 mmol/l |
| Phenol | 6 mmol/l |
| Peroxidase | ≥ 0.5 U/ml |
| Cholesterol esterase | ≥ 0.15 U/ml |
| Cholesterol oxides | ≥ 0.1 U/ml |
| Pipes Buffer | 80 mmol/l; pH 6.8 |

*Standard* 5.17 mmol/l (200 mg/dl)

***Materials***

Microcentrifuge tube

Micropipettes and pipettes

Disposable tips

AUTOLAB (Analyzer Medical system, Rome, Italy)

***Procedure***

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the AUTOLAB. 5 μl sample and 500 μl reagent were mixed and incubated at 370C for 5 minutes within the Auto lab. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

***Calculation of result***

Concentration of cholesterol in sample was calculated by using software program with the following formula.

Cholesterol concentration (mg/dl) = $\frac{A_{Sample}}{A_{Standard}}$ × concentration of standard.

## Appendix- V

**Estimation of serum Triglycerides**, UK (Trinder 1969)

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in auto analyzer (Analyzer Medical System, Rome, Italy) using reagent of Randox laboratories, UK (Trinder 1969)

### *Principle*

The triglyceride is determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen- peroxide, 4- aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

$$\text{Triglyceride} + H_2O \xrightarrow{Lipase} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + ATP \xrightarrow[Kinase]{Glycerol} \text{glycerol-3-phosphate} + ADP$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow[Oxidase]{Glycerol\ Phosphate} \text{dihydroxy acetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4- chlorophenol} \xrightarrow{Peroxidase} \text{quinoneimine} + HCl + 4\,H_2O$$

### *Reagents*

| *Contents* | *Concentrations in the Test* |
|---|---|
| Buffer | |
| Pipes Buffer | 40 mmol/l, pH 7.6 |
| 4-choloro-phenol | 5.5 mmol/l |
| Magnesium-ions | 17.5 mmol/l |
| 2. Enzyme Reagent | |
| 4-aminophenazone | mmol/l |
| ATP | 1.0 mmol/l |
| Lipases | >150 U/ml |
| Glycerol-3-phosphate oxidase | 1.5 U/ml |
| Peroxidase | 0.5 U/ml |
| 3. Standard | 2.29 mmol/l (200 mg/dl) |

### *Materials*

Micropipettes and pipettes

Disposable tips

AUTOLAB (Analyzer medical system, Rome, Italy)

### *Procedure*

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the AUTOLAB. 5 µl sample and 500 µl reagent were mixed and incubated at 370C for 5 minutes within the AUTOLAB.

The reaction occurred in reaction cell. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

### *Calculation of result*

Triglyceride concentration was calculated by using software program in AUTOLAB with the following formula.

$$\text{Triglyceride concentration (mg/dl)} = \frac{A_{Sample}}{A_{S\,tan\,dard}} \times \text{Concentration of the standard.}$$

## Appendix- VI

**Estimation of Serum High Density Lipoprotein** (HDL) (Analyzer Medical System, Rome, Italy)

Serum High density lipoprotein (HDL) was measured by enzymatic colorimetric (cholesterol CHOD-PAP) method in auto analyzer using reagent of Randox laboratories, UK (Friedewald WT, 1972)

### *Principle*

HDL (High Density Lipoproteins) are separated from chylomicrons, VLDL (very low density iipoproteins) and LDL (Low density lipoproteins) by the addition of a precipitating reagent (phosphotungstic acid-magnesium chloride) to serum or serum. After centrifugation, the cholesterol contents of HDL fraction, which remains in the supernatant, are determined by the enzymatic colorimetric method using CHOD- PAP.

### *Reagent composition*

Contents

Buffer

Enzymes

Standard 50 mg/dl (1.29 mmol/l)

### *Materials*

Microcentrifuge tube, Micropipettes and pipettes

Disposable tips

AUTOLAB

### *Procedure*

Samples (200 ml) and precipitating reagents (500 μl) were taken in a microcentrifuge tube. Then it was mixed and allowed to sit for 10 minutes at room temperature. Then it was centrifuged at 4000 rpm for 10 minutes.

The supernatant was used as sample for determination of cholesterol content by the CHOD-PAP method. The sample and reagents were taken in specific cup or cell. They were arranged serially then ID number for test was entered in the AUTOLAB. Then 5 μl sample and 500 μl reagent were mixed and incubated at 370C for 5 minutes within the AUTOLAB. The reaction occurred in reaction cell.

The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration was calculated by using software program.

## Appendix- VII

### Estimation of LDL-Choiesterol

The LDL-Cholesterol level in serum was calculated by using by Friedewald formula (Friedewald WT. 1972)

***Formula***

LDL cholesterol =Total cholesterol– (HDL Cholesterol + $\frac{1}{5}x$ Triglyceride)

## Appendix- VIII

**Estimation of Serum Insulin level by** Microparticle Enzyme Immunoassay (MEIA) technology:

The AxSYM Insulin assay is based on the Microparticle Enzyme Immunoassay (MEIA) technology.

***Principals***

The AxSYM Insulin Reagents and samples are pipetted in the following sequence:

Sample and all AxSYM Insulin assay reagents required for one test are pipetted by the Sampling Probe into various wells of the reaction vessel (RV) in the Sampling Center. The RV is immediately transferred into the Processing Center.

The Processing Probe does further pipetting in the processing Center. The reactions occur in the following sequence:

***Reagents:***

1 bottle (6.8 mL) Anti-Insulin (Mouse, Monoclonal) Coated Microparticles in buffer with protein stabilizers. Preservatives: Sodium Azide and Antimicrobial agents. (Reagent bottle 1).

1 bottle (11.8 mL) Anti-Insulin (Mouse, Monoclonal): Alkaline Phosphatase Conjugate in buffer with protein stabilizers. Minimum concentration: 3 µg/mL. Preservatives: Sodium Azide and Antimicrobial agents. (Reagent bottle 2).

1 bottle (14.4 mL) Assay Buffer in Calf serum. Preservatives: Sodium Azide and Antimicrobial agents. (Reagent bottle 3).

***Calibrators:***

AxSYM Insulin Master Calibrator (2D01-30):

The two bottles (4 mL each) of AxSYM insulin master calibrators contain insulin (human, porcine derivative) prepared in buffer at the following concentration

| **Bottle** | **Insulin concentration (µU/mL)** |
|---|---|
| MASTER CAL 1 | 0 |
| MASTER CAL 2 | 100 |

Preservatives: Sodium Azide and Antimicrobial agents.

***Standard Calibrators:***

The six bottles (4 mL each) of AxSYM Insulin Standard Calibrators contain insulin (human, porcine derivative) prepared in buffer at the following concentrations:

| **Bottle** | **Insulin concentration (µU/mL)** |
|---|---|
| STANDARD CAL A | 0 |
| STANDARD CAL B | 3 |
| STANDARD CAL C | 10 |
| STANDARD CAL D | 30 |
| STANDARD CAL E | 100 |
| STANDARD CAL F | 300 |

Preservatives: Sodium Azide and Antimicrobial agents.

***CONTROLS***

AxSYM Insulin controls (2D01-10):

The three bottles (8 mL each) of AxSYM Insulin Controls contain insulin (human, porcine derivative) prepared in buffer to yield the following concentration ranges:

| Bottle | Insulin concentration (µU/mL) | Range (µU/mL) |
|---|---|---|
| CONTROL L | 8 | 6-10 |
| CONTROL M | 40 | 32-48 |
| CONTROL H | 120 | 96-144 |

Preservatives: Sodium Azide and Antimicrobial agents.

***Other Reagents:***

Solution 1(MUP)

4 Bottles (230 mL each) Solution 1 containing 4-methylumbelliferyl Phosphate, 1.2 mM, in buffer. Preservative: Sodium Azide.

Solution 3(Matrlx Cell Wash)

4 Bottles (1000 mL each) Solution 3 (Matrix Cell Wash) containing 0.3 M Sodium Chloride in TRIS Buffer.

Preservatives: Sodium Azide and Antimicrobial agents.

Solution 4 Line Diluent

1 Bottle (10) Solution 4 (Line Diluent) containing 0.1 M Phosphate Buffer. Preservatives: Sodium Azide and Antimicrobial agents.

AxSYM Probe cleaning Solution:

4 Bottles (110 mL each) AxSYM Probe cleaning Solution containing 2% Tetraethyammoniumhydroxide (TEAH).

## Appendix- IX

**Estimation of Serum SHBG** (Sex Hormone Binding Globulin) by Chemiluminescent Immunometric Assay

***Principle***

IMMULITE/IMMULITE 1000 SHBG is a solid-phase, chemiluminescent immunometric assay.

***Volume required:***

10µL of the prediluted patient serum sample. (Sample cup must contain at least 100 µL more than the total volume required.)

***Reagents:***

Store at 2-8°C. Dispose in accordance with applicable laws.

**Water:** Use distilled or deionized water.

***Assay Procedure:***

Note that for optional performance, it is important to perform all routine maintenance procedures defined in IMMULITE or IMMULITE 1000 Operator's Manual.

For preparation, setup, dilution, adjustment, assay and quality control procedures the IMMULITE or IMMULITE 1000 Operator's Manual are seen.

All patient serum samples must be diluted 1-in-21 in SHBG Sample Diluent either manually (IMMULITE) or automatically, on-board (IMMULITE 1000 Windows®).

For automatic, on-dilutions (IMMULITE 1000 Windows®), The Reagent Wedge and the Diluent Wedge must be loaded on the carousel.

Each Test Unit was inspected for the presence of a bead before loading it into the system.

These limits are considered as guidelines only. Each laboratory should establish its own reference ranges.

***Limitations:***

Free androgen index: SGBH results should be interpreted in conjunction with measure of the hormones with which it binds, notably, testosterone. Combining such information in the form of a free androgen index (FAI)- computed as the ratio of total testosterone to SHBG –has been reported to yield better discrimination of women with hyperandrogenemic hirsutism from the healthy women than the use of SHBG levels on their own.

***SHBG controls:***

SHBG controls are assayed, bi-level controls intended for used with the IMMULITE/IMMULITE 1000 SHBG assay. They are intended ad an aid in monitoring day –to – day performance.

***Materials Supplied:***

**Control 1 and 2 (LSHC 1, LSHC 2)**

Two vials containing lyophilized SHBG in a nonhuman protein/buffer matrix, with a preservative. At least 30 minutes before use: reconstitute each vials with 2.0 mL distilled or deionized water. Mix by gentle inversion until the lyophilized material is fully dissolved.

Stable at 2-8°C for 30 days after reconstitution or for 6 months (aliquotted) at -20°C.

No 1-in -21 dilution is required after reconstitution for SHBG controls.

The controls are intended to be assayed as unknown, in the context of an internal quality control program. Each control should be identified by its lot number (read for the vial) and the detail it was opened/ reconstituted.

***Procedure:***

Assay according to the procedure provided in the appropriate package inserts. Enter controls through software "Control" function.

Target values, nmol/L

| Level | Mean | SD | 2SDRange |
|---|---|---|---|
| LSHC 10018 | 6.5 | 0.39 | 5.7-7.3 |
| LSHC 20018 | 98 | 5.5 | 87-109 |

***Expected Values:***

Based on its relationship to DPC's IRMA-Count SHBG the assay can be expected to have the following approximate reference ranges for adults with normal testosterone levels.

| Group | Central 95% nmol/L | Median nmol/L | n |
|---|---|---|---|
| Males | 13-71 | 32 | 122 |
| Female (nonpregnant) | 18-114 | 51 | 111 |

## Appendix- X

**Estimation of Fasting Serum Creatinine** (Randox Laboratories, UK).

Estimation of serum creatinine was done by alkaline-picrate methods using reagents

***Principle***

Creatinine in alkaline solution reacts with picric acid to form a colored complex. The amount of the complex formed is directly proportional to the Creatinine concentration

***Sample***

Serum.

***Reagents***

| | |
|---|---|
| Standard | 177umol/1 (2mg/dl) |
| Picric acid surfactant | 35 mmol/L |
| Sodium hydroxide | 0.32 mol/L |

***Preparation of reagent***

All reagents are supplied ready to use, stable to expiry date when stored at +15 to 250C.

***Preparation of working reagent***

Mix Equal volumes Of Solutions 2+3, Stable for 3 Days At + 15 to+250C.

***Procedure***

| | | |
|---|---|---|
| Wavelength | | 492 (490-510) mm |
| Cuvette | | 1 Cm Light Path |
| Temperature | | 25/30/37 C |
| Measurement | | against Air |
| Pipette into cuvette | Standard | Sample |
| Working reagent | 1.0 ml | 1.0 ml |
| Standard solutions | 0.1 ml | --- |
| Sample | | |

Mix and after 30 sec, read the absorbance A1 of the standard and sample

Exactly 2 min. later read the absorbance A2 of the standard and sample

***Calculation***

A2-A1 = ΔA sample or ΔA standard

Concentration of Creatinine in serum

$$\frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 100 = \text{mg/dl}$$

## Appendix- XI

### Estimation of Urine Total Protein

Pyrogallol red method for urinary total protein.

***Principle***

At PH 2.5 the formation of the complex between the pyrogallol red – molibdate and the proteins produces a change in the absorbance at 600 nm. This change is proportional to the protein concentration and can be measured spectrophotometrically.

***Reagents***

Kit (Ref. 99 21 00). Contents:

| | | |
|---|---|---|
| 2X100 ml | Pyrogallol red reagent. | (Ref 99 22 08) |
| 1X5 ml | Standard | (Ref 99 23 05) |

Equivalent to 200 mg of proteins/dl (2 g/l).

Concentrations in the reagent solution are:

| | |
|---|---|
| Succinate buffer pH 2.5 | 60 mM |
| Pyrogallol red | 0.4 mM |
| Sodium molybdate | 0.1 mM |
| Sodium oxalate | 1.2 mM |
| Sodium benzoate | 2.6 mM |
| Surfactants | 1 % |

***Procedure***

| **Procedure** | **BL** (ml) | **SA** (ml) | **ST** (ml) |
|---|---|---|---|
| Sample | -- | 0.02 | -- |
| Standard | -- | -- | 0.02 |
| Reagent | 1.00 | 1.00 | 1.00 |

Mix well and let stand for 10 min at room temperature (250C).

| **High sensitivity assay** | **BL** (ml) | **SA** (ml) | **ST** (ml) |
|---|---|---|---|
| Sample | -- | 0.05 | -- |
| Standard | -- | -- | 0.05 |
| Reagent | 3.00 | 3.00 | 3.00 |

**Reading**

Wavelength: 600 nm (580 – 620 nm).

Blank: BL Contents.

Color stability: 30 minutes.

***Calculation of results***

$$\frac{\text{SA O.D.}}{\text{ST O.D.}} \text{ X 2000 XL urine/24 h = mg prot/24 h}$$

To express the results in μg/min, multiply the mg/24 h by the factor: 0.6944:

(mg/ 24 h) X 0.6944 = μg/min.

***Normal values***

28 – 141 mg/24 h

19 – 98 μg/min.

## Appendix- XII

**Urine Protein (Albumin) By Uriscan Strip** (YD Diagnostics, Thailand).

Urine protein (albumin) test by Uriscan strip

Several rapid screening tests are in routine use. The majorities of the test strips have been developed to detect albumin and may be negative in the presence of other proteins, such as Bence Jones Proteins.

***Principle***

It is based on the protein error of a pH indicator. At a constant pH any color change that happens to an indicator is due to protein. The test area of the reagent strip is impregnated with an indicator, tetrabromophenol blue, buffered to pH 3.0. At this pH it is yellow in the absence of protein. Protein forms a complex with the dye turning the color of the dye to green or bluish green.

***Procedure***

Collect fresh, well-mixed, uncentrifuged urine specimen in a clean dry container.

Mix well immediately before testing.

Take strip(s) out from the container and replace the cap immediately.

Inspect the strip. If reagent areas are discolored, do not use strips.

Dip the test strip into the urine up to the mark for no more than 1 second.

Wipe off excess urine on with an absorbent paper. Lightly touch the edge of one side of the test strip on an absorbent paper.

Read test results carefully at 60 seconds in a good light and with the test area held near the appropriate color chart on the bottle label. Changes in color that appear only along the edges of the test patches or after more than 2 minutes

have passed are of no diagnostic significance. Reading with leucocytes test portion can be read at 120 seconds

***Specificity***

Protein Albumin

***Result***

The color is compared with the color chart provided, which indicates the approximate protein concentration.

Sensitivity and color blocks with concentrations

| **Test portion** | **Sensitivity** | **Color Chart** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **£** | **i¾** | **+** | **++** | **+++** | **++++** |
| Protein | 10mg/dL | 0 | 10 | 30 | 100 | 300 | 1000 |

A false-positive result may occur if:

The specimen is contaminated with vaginal or urethral secretions a strongly alkaline urine is used the urine container is contaminated with disinfectants such as chlorohexidine.

False-negative results will be observed if acid has been added to the urine as a preservative (for example in the estimation of urinary calcium).

## Appendix- XIII

### Estimation of urine creatinine

Estimation of urine creatinine by alkaline-picrate method (Randox, UK)

***Principle***

Creatinine in alkaline solution reacts with picric acid to form a colored complex. The amount of the complex formed is directly proportional to the creatinine concentration

***Sample***

The urine sample is diluted as the ratio of 1:49 with distilled water.

***Reagents***

| Contents | Initial Concentration of Solution |
|---|---|
| 1. Standard | 177μmol/1 (2mg/dl) |
| 2. Picric acid surfactant | 35 mmol/L |
| 3. Sodium hydroxide | 0.32 mol/L |

***Preparation of reagent***

All regents are supplied ready to use, stable to expiry date when stored at + 15 to 25° c.

***Preparation of working reagent***

Mix Equal Volumes Of Solutions 2+3, Stable For 3 Days At + 15 to+25°C

***Procedure***

| | |
|---|---|
| Wavelength | Hg 492 (490-510mm) |
| Cuvette | 1 Cm Light Path |
| Temperature | 25/30/37 C |
| Measurement | Against Air |

| Pipette into cuvette | Standard | Sample |
|---|---|---|
| Working reagent | 1.0 ml | 1.0 ml |
| Standard solutions | 0.1 ml | --- |

Sample 0.1 ml

Mix and after 30 sec, read the absorbance A1 of the standard and sample. Exactly 2 min. later read the absorbance A2 of the standard and sample.

***Calculation***

**A2 –A1** = ΔA sample or ΔA standard

Concentration of creatinine in urine

$$\frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 100 = \text{mg/dl}$$

Positive Predictive values were calculated by considering urinalysis of ≥1+ (0.3 g/l) to be positive and measured urine sample protein concentration of ≥ 0.3 g/l to be true positive. Negative Predictive values were calculated by considering urinalysis of nil or trace to be negative and urine sample protein concentration of < 0.3 g/l to be true negative. Specificity and sensitivity were also determined on this basis. Differences in the frequency of positive and negative results were calculated by Chl-squlre testing using Macnamer test.

## Appendix- XIV

# Calculation of B cell function and insulin sensitivity

Homeostasis Model Assessment (HOMA) is a simple widely used method which derives separate indices of B cell secretion (HOMA B) and insulin sensitivity (HOMA S) from the plasma glucose and insulin concentrations under basal condition by using mathematical formula or software. Using HOMA, insulin sensitivity (HOMA-IR) is calculated as [fasting C-peptide (ngm/ml) x fasting glucose (mmol/l)/22.5]. The HOMA index of insulin secretion (HOMA B-cell) is calculated as fasting insulin (mu/l) x 20/ [fasting glucose (mmol/l)-3.5][37] The HOMA model has been incorporated in a simple MS-DOS-based computer program (HOMA-CIGMA software) that allows rapid determination of %B (B cell secretion) and %S (insulin sensitivity) from measured values using fasting plasma glucose in mmol/l and insulin in pmol/l. Although the simple equation gives a qualitatively useful approximation of the model prediction, most authors prefer the computer model. In this study HOMA-CIGMA software was used.

## Appendix- XV

### *Quantitative insulin sensitivity check index (QUICKI).*

It is derived by calculating the inverse of the sum of logarithmically expressed values of fasting glucose and insulin:

$$\frac{1}{[\log(I0) + \log(G0)]}$$

## Appendix- XVI

### Estimation of UPr/Cr ratio

The urine protein-creatinine ratio was obtained by dividing the urine protein concentration (mg/l) by the urine creatinine concentration (mol/l) 13. True proteinuria (≥ 300mg/day) was detected UPr/Cr ratio of > 30 mg protein/mmol creatinine.

Sensitivity, specificity, positive and negative predictive values were determined for different UPr/Cr ratio values for proteinuria > 30 mg protein/mmol creatinine as positive and < 30 mg protein/mmol creatinine as negative.