The Association of Trace and Ultratrace Elements with Pre-eclampsia

Preeclampsia, a highly morbid disease, affect about six per cent of pregnant women in UK with tendency toward primigravid women. A lot of theories has been postulated regarding its etiology and pathogenesis including genetic, immunological, vascular and nutritional factors. In the last years, disturbance in homeostasis of trace and ultra trace elements has been implemented in the etiology of preeclampsia. This book discuss the possible mechanisms through which these elements might cause preeclampsia, method of measuring them and difference in their blood level when comparing preeclamptic patients with healthy pregnants.

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Trace Elements' Role in Pre-eclampsia

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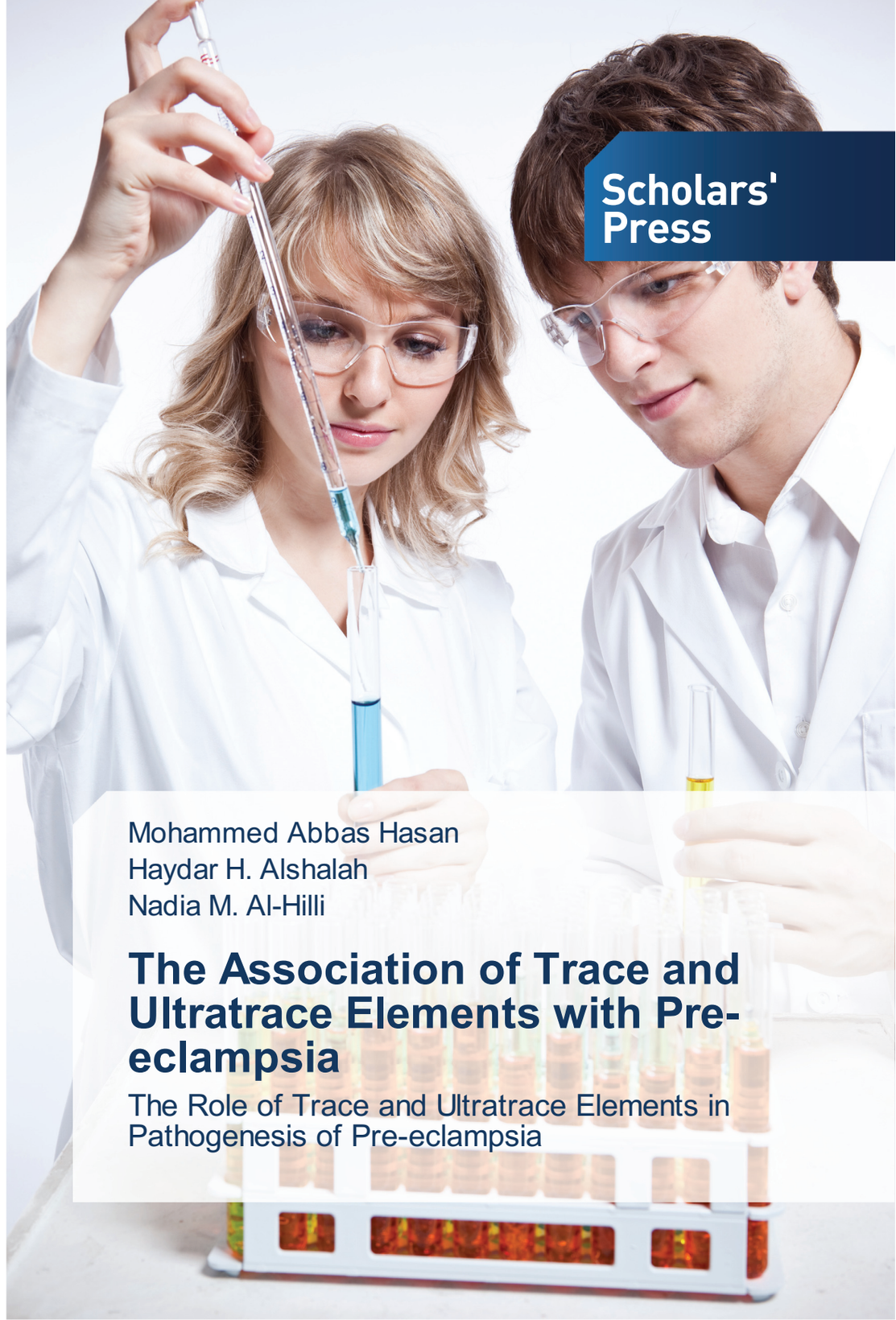

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Summary

Preeclampsia is defined as hypertension associated with proteinuria arising de novo after the 20th week of gestation in a previously normotensive woman and resolving completely by the 6th postpartum week. It is a major cause of morbidity and mortality during pregnancy. In UK, preeclampsia affects 3-5% of pregnancies. Its aetiology remains incompletely understood, and is considered as a disease of theories. One of these theories refers to the effect of heavy metals, trace, and ultra trace elements in the corresponding patients. In the present study some trace and ultra trace elements were estimated to identity their role in the pathogenesis of pre-eclampsia. This study was carried out in Babylon Teaching Hospital for Gynecology and Pediatrics, in Babylon Province, Hilla City. All samples were collected from November 2014 till February 2015. This is a case control study which included 120 women, sixty were patients diagnosed with preeclampsia in the third trimester and the other sixty were healthy pregnant women (controls) in the third trimester. Cases with age over 40, BMI > 30, previous history of pre-eclampsia, family history of pre-eclampsia, multiple pregnancy and hydrops fetalis, pre-existing hypertension or renal disease, pre-existing vascular disease, antiphospholipid syndrome, and smoking were excluded. Serum levels of iron, zinc, magnesium were measured by using a colorimetric method, while serum concentrations of copper, chromium, cobalt, manganese, molybdenum, and selenium were measured by using graphite furnace atomic absorption spectrophotometric technique.

The results were expressed as mean ± standard error of mean. T-test and the linear regression analysis were used for the determination of the level of significance. Statistical analysis were performed with Statistical

Package for the Social Sciences (SPSS) version 21.0 software. A P value of < 0.05 was considered to be statistically significant.

Serum total iron level was significantly higher in patients with pre-eclampsia compared to control group (186.498 µg/dl versus 94.392 , P value < 0.05). While no significant difference was found in molybdenum level between them (2.304 µg/dl versus 2.670, P value > 0.05). Finally, serum total concentrations of the other elements were significantly lower in patients with pre-eclampsia compared to control group as illustrated below:

- Copper (143.153 µg/dl versus 209.657 µg/dl, P value < 0.05)
- Chromium (0.382 µg/dl versus 0.678 µg/dl, P value < 0.05)
- Cobalt (0.143 µg/dl versus 0.330 µg/dl, P value < 0.05)
- Magnesium (2.115 mg/dl versus 2.456 mg/dl, P value < 0.05)
- Manganese (7.617 µg/dl versus 10.847 µg/dl, P value < 0.05)
- Selenium (2.546 µg/dl versus 4.306 µg/dl, P value < 0.05)
- Zinc (57.283 µg/dl versus 87.535 µg/dl, P value < 0.05).

In conclusion, alteration in the levels of serum trace and ultra trace elements could contribute to the pathogenesis of pre-eclampsia.

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Abbreviations

ABBREVIATION	DETAILS
5-Br-PAPS	2-(5-Brom-2-pyridylazo)-5-(N-propyl-N-sulfopropyl amino)-phenol
AMP	Adenosine monophosphate
A_s	Absorbance of sample
A_{st}	Absorbance of standard
ATP	Adenosine triphosphate
ATP7A	Menkes copper transporter ATP7A
ATP7B	Copper-transporting P-type adenosine triphosphate
BMI	Body mass index
BP	Blood pressure
ºC	Centigrade
CAB	Chromazurol B
CNS	Central nervous system
CTMA	Cetyltrimethyl-ammonium bromide
Cu-Zn SOD	Copper-zinc super oxide dismutase
dl	Deciliter
DMT 1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
EGTA	Ethylene glycol tetra acetic acid
FAAS	Flame atomic absorption spectrometry
FAD	Oxidized flavin adenine dinucleotide
GFAAS	Graphite furnace atomic absorption spectrometry
HDL-Cholesterol	High density lipoprotein cholesterol

HELLP Syndrome	Hemolysis, elevated liver enzymes, low platelet syndrome
HLA-A,-B ,-D, la, -ll	Human leukocyte antigens -A, -B, -D, la, -ll
ICP-AES	Inductively coupled plasma atomic emission spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IL	Interleukins
ISSHP	International Society for the Study of Hypertension in Pregnancy
KCN	Potassium cyanide
KIR	Killer- immunoglobulin-Like receptors
mA	Milli Amper
mmHg	Millimeter of mercury
mmol	Millimole
NADH	Reduced nicotinamide adenine dinucleotide
ng	Nanograms
NICE	National Institute for Health and Care Excellence for use in pregnancy
NK-Cell	Natural killer cell
nm	Nanometer
O.D.	Optical density
$O_2^{-\bullet}$	Superoxide anion
OH^-	Hydroxyl anion
$OH^\cdot$	Hydroxyl radical
P. Value	Probability

PE	Pre-eclampsia
ppb	Parts per billion
ppm	Parts per million
PTH	Parathyroid hormones
Q.S.	Quaternary Surfactant
RBC	Red blood cell
RE System	Reticuloendothelial system
Redox reaction	Oxidation - reduction reaction
ROS	Reactive oxygen species
SEM	Standard error of mean
sFLT-1	Soluble FMS-like tyrosine kinase-1
SPSS	Statistical Package for the Social Sciences
TNF-α	Tumour necrosis factor-α
V	Volume of solution

CHAPTER ONE

Introduction

1. INTRODUCTION

1.1. General Introduction

Pre-eclampsia is an idiopathic disorder of pregnancy characterized by proteinuria and hypertension. Recent studies indicate that over 63000 women die worldwide each year due to pre-eclampsia and its complications, with 98% of them occurring in developing countries **[1]**. In UK, pre-eclampsia is the second most common cause of both direct maternal death and perinatal loss, responsible for the death of six to nine women annually and over 175 babies **[2]**. More than 10% of women will have pre-eclampsia in their first pregnancy and although the majority of these will have successful pregnancy outcomes, the condition can give rise to severe multisystem complications including cerebral haemorrhage, hepatic and renal impairment and respiratory compromise. The development of strategies to prevent and treat the disorder has been challenging due to an incomplete understanding of the underlying pathogenesis **[3].**

1.2. Proteinuria

It is defined as one of the following:

- One 24-h urine collection with a total protein excretion of 300 mg or more; or
- Two random clean-catch urine specimens with a 2+ or more on reagent strip **[4].**

1.3. Hypertension

Hypertension in pregnancy is defined as one of the following:

- One measurement of diastolic BP of 110 mmHg or more ; or

- Two consecutive measurements of diastolic BP of ≥ 90 mmHg 4 hours or more apart with the patient at rest **[5].**

1.3.1. Classification of Hypertensive Disorders During Pregnancy

Pregnant women with hypertension can be broadly divided into one of three categories: chronic hypertension, non-proteinuric gestational hypertension (also known as gestational hypertension) and pre-eclampsia. It's important to distinguish between them as management and prognosis are different. The International Society for the Study of Hypertension in Pregnancy (ISSHP) uses the term gestational hypertension in women who were previously normotensive.

1.3.2. The Classification of International Society for the Study of Hypertension in Pregnancy (ISSHP):

A. Gestational hypertension and/or proteinuria developing during pregnancy, labour or the puerperium in a previously normotensive non-proteinuric woman

1. Gestational hypertension (without proteinuria).
2. Gestational proteinuria (without hypertension).
3. Gestational proteinuric hypertension (pre-eclampsia).

B. Chronic hypertension (before the 20^{th} week of pregnancy) and chronic renal disease (proteinuria before the 20^{th} week of pregnancy).

1. Chronic hypertension (without proteinuria).
2. Chronic renal disease (proteinuria with or without hypertension).
3. Chronic hypertension with superimposed pre-eclampsia (new onset proteinuria).

C. Unclassified hypertension and/or proteinuria.

D. Eclampsia **[6]**.

1.3.3. Gestational Hypertension

Hypertension arising for the first time after the twentieth week of gestation, in the absence of proteinuria (< 300 mg in a 24-hour urine collection), has neither a significant maternal nor fetal consequences. Blood pressure returns to normal by 6 weeks postpartum or before this time **[7,8]**.

1.3.4. Chronic Hypertension

Hypertension which is apparent prior to, in the first half of, or persisting more than 6 weeks after pregnancy **[9]**.

1.3.5. Pre-eclampsia Superimposed on Chronic Hypertension

Chronic hypertension in pregnancy may be complicated by preeclampsia. This condition will be determined when there is a new outset of proteinuria or sudden deterioration of either hypertension or proteinuria, or evolution of other signs and symptoms of preeclampsia after the twentieth week of gestation **[8,10]**.

1.3.6. Pre-eclampsia

Preeclampsia is defined as hypertension associated with proteinuria arising de novo after the 20^{th} week of gestation in a previously normotensive woman and resolving completely by the 6^{th} postpartum week . It is a leading cause of fetal and maternal morbidity and mortality **[12]**.

1.3.6.1. Eclampsia

It is a serious life-threatening complication of pre-eclampsia when tonic-clonic convulsion occur in a woman with established pre-eclampsia, in the absence of any other neurological or metabolic cause.

1.3.6.2. Severe Pre-eclampsia

It is a pre-eclampsia with severe hypertension and/or with symptoms of imminent eclampsia, and/or biochemical and/or hematological impairment.

1.3.6.3. Incidence of Pre-eclampsia

Hypertensive disorders affect about 6-12% of pregnant women **[3]**. In UK, preeclampsia affects 3-5% of pregnancies **[12]**.

1.3.6.4. Risk Factors for Pre-eclampsia

- First pregnancy.
- Multiparous with:
  - ❖ pre-eclampsia in any previous pregnancy,
  - ❖ ten years or more since last pregnancy.
- Age 40 years or more.
- Body mass index of 35 or more.
- Family history of pre-eclampsia (in mother or sister).
- Booking diastolic blood pressure of 80 mmHg or more.
- Booking proteinuria (of $\geq$ 1+ on more than one occasion or quantified at $\geq$ 0.3 g/24 hour).
- Multiple pregnancy.
- Certain underling medical conditions :
  - ❖ pre-existing hypertension.
  - ❖ pre-existing renal disease.
  - ❖ pre-existing diabetes.
  - ❖ antiphospholipid antibodies **[13] [14]**.

1.3.6.5. Aetiology and Pathogenesis of Pre-eclampsia

The aetiology remains incompletely understood, and many theories have been considered.

Factors that are likely to be involved in the aetiology of this disease are :

- Placental implantation with abnormal trophoblastic invasion of uterine vessels.

- Immunological maladaptive tolerance between maternal, placental and fetal tissues.
- Maternal maladaptation to cardiovascular or inflammatory changes of normal pregnancy.
- Genetic factors including inherited predisposing genes as well as epigenetic influences.
- Nutritional factors **[15]**.

1.3.6.5.1. Abnormal Trophoblastic Invasion:

In normal placental implantation, the extra villous trophoblast proliferates from an anchoring villous, as shown in Figure 1-1. The cytotrophoblast invade the uterine spiral arteries in the inner part of the myometrium. Invasion of the spiral arteries is associated with degeneration of the tunica media and replacement by fibrinoid material resulting in loss of resistance and marked dilatation of the spiral artery and increased intervillous blood flow [16].

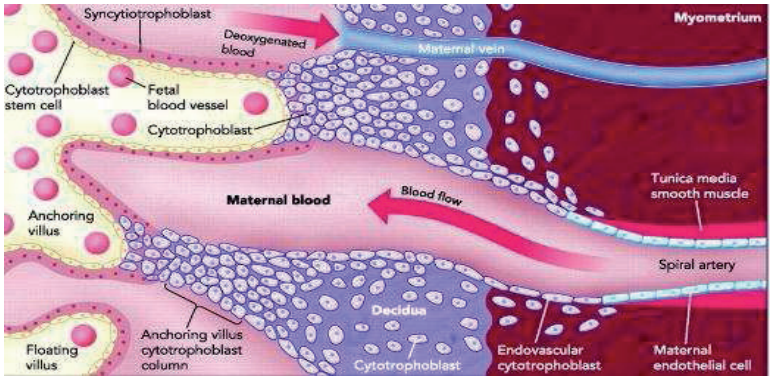

Figure (1.1): Normal placental implantation [17].

In pre-eclamptic patients, the placenta suffers from defective implantation, as shown in Figure 1-2. The disease process is composed of two phases. The first phase is characterized by patchy trophoblast invasion so that the spiral arteries retain their muscular walls which will prevent the

development of high-flow, low-impedence uteroplacental circulation. The pre-eclamptic placenta will get high resistance, so the flow of blood will be decreased and the perfusion will be inefficient. These will cause ischemia and hypoxia of the placenta. This probably results in the production of reactive oxygen species. Once the normal endogenous antioxidants are overwhelmed, a condition of oxidative stress exists. This is probably fundamental to the clinical syndrome of pre-eclampsia **[13]**.

Either through oxidative stress or other vasoactive substances being released from the placenta, activation of the vascular endothelium all over the body will occur. In the second phase of pre-eclampsia, all maternal organs are affected by the general vascular endothelial dysfunction **[18]**.

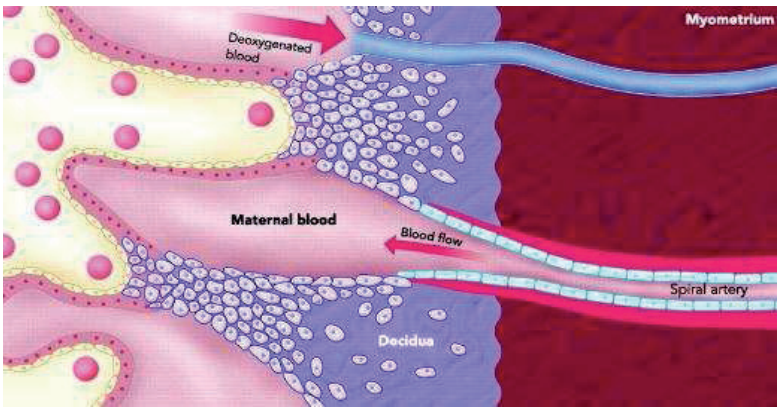

Figure (1.2): Placenta in pre-eclamptic or fetal growth restricted pregnancy [17].

1.3.6.5.2. Immunological Changes

There is maternal immune tolerance to parental derived placental and fetal antigens. Loss of this tolerance or perhaps its disregulation leads to pre-eclampsia. There are many inferential data that suggest an immune –mediated disorder. As a risk of pre-eclampsia is appreciably increased in circumstances

where formation of blocking antibodies to placental antigenic sites might be impaired. In this case, the first pregnancy would carry a higher risk **[19]**. Some women have elevated serum levels of antiangiogenic factors. The gene for one of these factors, soluble FMS-like tyrosine kinase-1(sFLT-1), is on chromosome 13 **[20]**. There are inherited immunogenetic factors that may modify genotype and phenotype expression in PE. Some examples of these factors are shown in table (1-1) **[21]**.

Table (1-1) : Some examples of inherited immunogenetic factors.

Immunization from a prior gestation.
Inherited haplotypes for HLA-A,-B ,-D, la, -ll
Inherited haplotypes for NK- cell receptors-also called Killer-immunoglobulin-Like receptors-KIR.
Possibly shared susceptibility genes with diabetes and chronic hypertension.

1.3.6.5.3. Inflammatory Factors:

It has been proposed that endothelial cell dysfunction is due to an extremely activated state of leukocytes in the maternal circulation **[22]**. In short, cytokines such as tumour necrosis factor-α (TNF-α) and the interleukins (IL) may contribute to the oxidative stress associated with PE. This is characterized by reactive oxygen species and free radicals that lead to formation of self-propagating lipid peroxides. These in turn generate highly toxic radicals that cause injury of endothelial cells and modify their nitric oxide production and interfere with prostaglandin balance. Other consequences of oxidative stress include production of lipid–laden macrophage foam cells seen in atherosis; activation of macrovascular coagulation manifest by thrombocytopenia; and, increased capillary permeability manifested by oedema and proteinuria **[23]**.

1.3.6.5.4. Genetic Factors

The incidence risk of PE is (20% to 40%) for daughters of pre-eclamptic mothers; 11 to 37 percent for sisters of pre-eclamptic women; and 22 to 47 percent in twin pregnancies **[24]**.

1.3.6.5.5. Nutritional Factors

In general, diet rich in fruits and vegetables that have antioxidant activity is associated with decreased blood pressure **[25]**. The incidence of PE was doubled in women whose daily intake of ascorbic acid was less than 85 mg. These studies were followed by randomized trials to study dietary supplementation **[26]**. Calcium supplementation in populations with a low dietary calcium intake had a small effect to lower perinatal mortality rates, but no effect on the incidence of PE. In a number of trials, supplementation with antioxidant vitamins C and E showed no beneficial effect **[27]**.

1.3.6.6. Pathophysiology of Pre-eclampsia

Vasospasm and endothelial cell dysfunction, with subsequent platelet activation and micro-aggregate formation, account for many of the pathological features of pre-eclampsia seen in almost every major organ system **[13]**.

1.3.6.6.1. Cardiovascular System

Pre-eclampsia is characterized by lack of normal intravascular volume expansion, a reduction in normal circulating blood volume, and a loss of normal refractoriness to endogenous vasopressors, including angiotensin II **[28]**.

1.3.6.6.2. Central Nervous System (CNS)

In the brain, oedema and cerebral vasospasm, ischemia, and ionic shifts between intracellular and extracellular compartments are believed to incite eclamptic seizures. A new onset headache and increased reflex irritability or

hyperreflexia are extremely concerning signs of CNS involvement and may show imminent seizures **[29]**.

1.3.6.6.3. Liver Function

Abnormal liver function tests and elevation of liver enzymes above the normal non-pregnant reference ranges associated with PE may reflect liver dysfunction secondary to vasoconstriction in the hepatic vein. Histopathological examination of the liver in pre-eclampsia reveals periportal fibrin deposition, hemorrhage, and hepatocellular necrosis. Subcapsular hematoma is one of the most severe sequelae of severe PE and HELLP Syndrome (hemolysis, elevated liver enzymes, and low platelet) **[30]**.

1.3.6.6.4. Renal System

In the kidney, a highly characteristic lesion called 'glomeruloendotheliosis' is seen. This is relatively specific for PE (it is not seen with other hypertensive disorder) and is associated with impaired glomerular filtration and selective loss of intermediate weight proteins, such as albumin and transferrin, leading to proteinuria. Which causes a reduction in plasma oncotic pressure and exacerbates the development of oedema **[13]**.

1.3.6.6.5. Coagulation System

In Pre-eclampsia, organ perfusion is further comprised by activation of the coagulation cascade. Altered platelet function is seen in most women with PE. In normal pregnancy, there is increased biosynthesis of eicosanoids, particularly prostacyclin and thromboxane A_2. Prostacyclin is a vasodilator with platelet- inhibitory properties and thromboxane A_2 is a vasoconstriction with a tendency to promote platelet aggregation. Prostacyclin and thromboxane A_2 usually increase in proportion to one another and consequently there is a net neutralization, and homeostasis is maintained. In women with preeclampsia, this homeostasis is disrupted due to a relative

deficiency of prostacyclin. This occurs either because of a reduction in prostacyclin synthesis or because of an increased production of thromboxane A_2. This imbalance leads to platelet stimulation and also vasoconstriction and hypertension **[31]**.

1.3.6.7. Symptoms and Signs of Pre-eclampsia:

- May be asymptomatic,
- Headache,
- Visual disturbances,
- Epigastric and right upper abdominal pain,
- Elevation of blood pressure,
- Fluid retention (non-dependent oedema),
- Oedema (progressive) **[32]**.

1.3.6.8. Symptoms and Signs of Severe Pre-eclampsia:

- Frontal headache,
- Visual disturbances (blurred vision and flashing lights),
- Epigastric pain,
- General malaise and nausea,
- Restlessness,
- Agitation,
- Hyper-reflexia and clonus,
- Facial (especially periorbital) oedema,
- Right upper quadrant tenderness,
- Poor urine output,
- Papilloedema **[13]**.

1.4. The Mineral Elements

The mineral elements constitute only a small proportion of the body weight. There is a wide variation in their body content. For instance, calcium constitutes about 2% of body weight while cobalt about 0.00004%.

1.4.1. Classification of Mineral Elements

The body's minerals are classified as principal elements (macrominerals) and trace elements (microminerals). The seven principal elements constitute 60-80% of the body's inorganic material. These are calcium, phosphorus, magnesium, sodium, potassium, chloride and sulfur **[33]**. The principal elements are required in amounts greater than 100 mg/day. The trace elements are required in amounts less than 100 mg/day **[34]**.

The term "trace element" was originally used to describe the residual amount of inorganic analyte quantitatively determined in a sample. More sensitive analytical methods now provide accurate determination of most inorganic micronutrients present at very low concentrations in body fluids and tissue. Those present at (µg/dl) in body fluids and in tissue (mg/kg) are however still widely referred to as trace elements and those found at ng/dl or µg/kg as the "ultratrace elements." The corresponding dietary requirements are quoted in mg/day or µg/day, respectively **[35]**.

1.4.2. Categories of Trace Elements:

- Essential trace elements: Iron, copper, iodine, magnesium, manganese, zinc, molybdenum, cobalt, fluorine, selenium and chromium.
- Possibly essential trace elements: Nickel, vanadium, cadmium and barium.
- Non-essential trace elements: Aluminium, lead, mercury, boron, silver, bismuth, etc. **[33]**.

Pregnancy is a period of rapid growth and cell differentiation for both the mother and fetus. Consequently, it is a period during which both are vulnerable to changes in dietary supply, especially of those micronutrients that are marginal under normal circumstances. Essential trace elements are involved in various biochemical pathways **[36]**. Their specific and the most important functions are the catalytic role in chemical reactions and in structural function in large molecules such as enzymes and hormones **[37]**. Alterations in concentrations and homeostasis of each of these micronutrients in body are well-known contributors in pathophysiology of various disorders and disease **[36]**. Trace elements such as zinc (Zn), selenium (Se) and copper (Cu) display antioxidant activity, while others such as calcium (Ca) and magnesium (Mg) are essential micronutrients **[38]**. Despite several studies on pre-eclampsia, its aetiology has not yet been fully elucidated. Some studies have shown that changes in the levels of serum trace elements in pre-eclamptic patients may implicate its pathogenesis **[39]** while others have failed to show an association of serum levels of trace elements and prevalence of pre-eclampsia **[40]**.

It has been reported that there is an increased incidence of pre-eclampsia in zinc-deficient regions and it was later found that zinc supplementation reduced the high incidence of the disease. Furthermore, decreased levels of zinc, selenium and copper have been observed in patients with pre-eclampsia. Ugwuja and *et al*. reported that only copper was found to be statistically significant. The previous study on serum calcium and magnesium levels in pregnant women showed that there is significant difference between patients with pre-eclampsia and normal control group **[41]**.

1.4.3. Individual Trace Elements

1.4.3.1. Iron (Fe):

1.4.3.1.1. Functions of Iron:

Iron mainly exerts its functions through the compounds in which it is present. Hemoglobin and myoglobin are required for the transport of O_2 and CO_2. Cytochromes and certain non-heme proteins are necessary for electron transport chain and oxidative phosphorylation. Peroxidase, the lysosomal enzyme, is required for phagocytosis and killing of bacteria by neutrophils. Iron is associated with effective immunocompetence of the body **[33]**.

1.4.3.1.2. Dietary Sources of Iron:

- Exogenous: Foods rich in iron include:
  - ❖ Animal Sources: Meat, fish, liver, spleen, red marrow are very rich sources (2.0 to 6.0 mg/100 gm). Also it is found in shellfish.
  - ❖ Vegetable Sources: Cereals (2.0 to 8.0 mg/100 gm) are the major rich source. Legumes, molasses, nuts, amaranth leaves, and dates are other good sources.
- Endogenous: Fe is utilised from ferritin of Reticuloendothelial (RE) System and intestinal mucosal cells. Fe obtained from "effete" red cells is also reutilised **[42]**.

1.4.3.1.3. Absorption, Transport, and Metabolism of Iron:

The iron exist in two oxidized states ferric (Fe^{+3}) and ferrous (Fe^{+2}) ionic forms. The intestinal mucosal cells take up ferric form of iron during its absorption. Ferritin is the storage form of iron. Free iron acts as prooxidant agent and it is released from ferritin by the reducing agents that convert Fe^{3+} into Fe^{2+}. Under stress or pathological conditions, it undergoes Fenton reaction and Haber – Weiss reaction to generate Reactive Oxygen Species

(ROS), which in turn damage the biological macro molecules. Transferrin, the iron transfer protein may also undergo glycation due to stress or pathological conditions causing increased free iron levels. Glycated transferrin also enhances the production of free oxygen radicals such as hydroxide which amplify the oxidative effects of iron **[43]**.

1.4.3.1.4. Clinical Significance of Iron:

In pre-eclampsia, when tissues become ischemic, ROS such as superoxide and hydrogen peroxide are produced, but these ROS may not be able to initiate any cellular damage directly. The transition of metal ions such as iron, arising from ischemic placenta by destruction of red blood cells (RBCs) from thrombotic, necrotic and hemorrhagic areas can generate highly reactive hydroxyl radical by Fenton reaction. This radical can initiate lipid peroxidation, which if uncontrolled, results in endothelial cell damage .

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$ (Fenton reaction)

$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$

$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^-$ (HABER–WEISS REACTION) **[44]**.

1.4.3.2. Zinc (Zn):

1.4.3.2.1. Functions of Zinc:

Zinc is important for the activity of a number of enzymes like carbonic anhydrase, alkaline phosphatase, alcohol dehydrogenase, porphobilinogin synthase, leucine amino peptidase, carboxy peptidase, aldolase in glycolysis, DNA and RNA polymerases as zinc has crucial role in DNA synthesis **[45]**. Release of vitamin A from liver requires zinc **[46]**. Retinene reductase (zinc enzyme) participates in the regeneration of rhodopsin (visual cycle) **[47]**. Insulin is secreted, stored as a complex of zinc. Zinc is important for wound healing **[45]**.

1.4.3.2.2. Dietary Sources of Zinc

Good sources for zinc are liver, milk, fish, dairy products, cereals, legumes, oil seeds, yeast and spinach, etc. **[42]**.

1.4.3.2.3. Absorption, Transport, Metabolism, and Excretion of Zinc

It is absorbed in duodenum and ileum **[48]**. Absorption of zinc from the intestine appears to be controlled in a manner similar to iron. It is transported bound to a protein (α2-macroglobulin and transferrin). It is excreted in urine and feces **[49]**. Diets rich in calcium, phosphates interfere with Zn absorption **[50]**. It is bound as complex of protein metallothionein. The sulfur groups of the protein chelate zinc **[51]**.

1.4.3.2.4. Clinical Significance of Zinc:

Alteration of zinc (Zn) may play a role in the several diseases **[52]**. Normal homeostasis of Zn is regulated by the actions of Zn transporters like zinc-related protein. These transporters control the level of Zn inside and outside the cell **[53]**. It has been shown that zinc performs a considerable role for optimal function of more than 300 different enzymes **[54]**. Some studies have indicated that decreased zinc concentration is associated with fetal malformations, fetal growth restriction, preterm delivery, pre-eclampsia, and bleeding after delivery [55,56]. Serum or placental Zn levels have been shown to be low **[57]** or without change **[58]** in pre-eclamptic women. It has been revealed that concentrations of zinc and copper are reduced in pre-eclamptic patients **[59]**. While study of Ugwuja et al. **[60]** showed that only copper was statistically different. There are also contradictory studies on the relationship between serum trace element levels and event of pre-eclampsia **[39]**. Studies have indicated the possible association of trace elements with fetal growth

and development and its relationship to newborn body weight, neonatal morbidity and mortality **[61]**.

1.4.3.3. Magnesium (Mg):

1.4.3.3.1. Functions of Magnesium:

Magnesium is required for the formation of bones and teeth **[62]**. Mg^{2+} serves as a cofactor for several enzymes requiring ATP, e.g. hexokinase, glucokinase, phosphofructokinase, adenylate cyclase **[63]**. Mg^{2+} is necessary for proper neuromuscular function. Low Mg^{2+} levels lead to neuromuscular irritability **[64]**.

1.4.3.3.2. Dietary Sources of Magnesium:

Magnesium is widely distributed in plant and animal foods and in beverages. Green leafy vegetables, such as spinach, legumes, nuts, seeds, and whole grains, are good sources **[65]**.

1.4.3.3.3. Absorption, Transport, Metabolism, and Excretion of Magnesium:

Magnesium is absorbed by the intestinal cells through a specific carrier system **[66]**. Consumption of large amounts of calcium, phosphate and alcohol diminishes Mg absorption. Para thyroid hormones increase Mg absorption **[67]**. Magnesium is lost from the body in faeces, sweat and urine **[68]**.

1.4.3.3.4. Clinical Significance of Magnesium:

Decrease in serum magnesium levels than the normal range is called as hypomagnesaemia. Magnesium is present in most common food stuffs, low dietary intakes of magnesium are associated with nutritional insufficiency, accompanied by intestinal malabsorption, severe vomiting, diarrhea or other

causes of intestinal loss. Symptoms of hypomagnesaemia are impaired neuromuscular function, tetany, hyper irritability, and tremor, convulsions and muscle weakness **[69]**.

Increase in serum magnesium more than the normal levels is called as hypermagnesaemia. It is uncommon but is occasionally seen in renal failure. It is rarely caused by intravenous injection of magnesium salts and adrenocortical hypofunction. The most common manifestation of hypermagnesaemia is depression of the neuromuscular system **[70]**.

1.4.3.4. Copper (Cu):

Copper (Cu) is an important essential trace element for humans and animals. In biological systems, copper shifts between the cuprous (Cu^{1+}) and cupric (Cu^{2+}) forms, though the majority of the body's copper is in the Cu^{2+} form. The ability of copper to easily exchange between these ions explains its important role in oxidation-reduction (redox) reactions and in scavenging free radicals **[71]**.

1.4.3.4.1. Functions of Copper

Copper has been found to be an important constituent of vital Cu-dependent enzymes such as lysyl oxidase, cytochrome oxidase, tyrosinase, dopamine-β-hydroxylase, peptidylglycine alpha-amidating monooxygenase, monoamine oxidase, ceruloplasmin (copper oxidase), and copper-zinc superoxide dismutase (Cu-Zn SOD), functioning as antioxidants and as oxidoreductases and these enzymes act as antioxidant defense system **[72]**.

Thus as a part of powerful antioxidant it helps to protect the cell from damage. Copper is also present in ceruloplasmin and promotes the absorption of iron from the gastrointestinal tract **[73]**.

Copper deficiency is rare, but cases have been identified in human, which manifested as neutropenia, anemia and skeletal abnormalities with

atherogenic and electrocardiographic irregularities and is linked to low birth weight of neonates **[74]**.

1.4.3.4.2. Dietary Sources of Copper

Average diet provides 2 to 4 mg/day in the form of meat, shellfish, legumes, nuts and cereals **[42]**.

1.4.3.4.3. Absorption, Transport, Metabolism, and Excretion of Copper

Not all dietary copper is absorbed, the proportion varying from 35% to 70% for reasons that are still not fully understood, although an increased intake of zinc reduces absorption. From the gut, copper is carried to the liver bound to albumin and there it is incorporated into caeruloplasmin. Caeruloplasmin is then secreted into the blood and accounts for 80–90% of the circulating copper. The main route for excretion of copper is in the bile, with very little in the urine. Normal copper homoeostasis, therefore, depends on the balance between intestinal absorption and biliary excretion **[75]**.

1.4.3.4.4. Clinical Significance of Copper

Copper can produce the highly reactive hydroxyl radical. The generation of this radical can cause lipid peroxidation process which may cause endothelial cell damage **[76]**.

Many studies have shown that copper concentration is increased in pre-eclamptic patients **[77]**.

1.4.3.4.4.1. Inborn Errors of Copper Metabolism:

- Wilson's disease (Hepatolenticular Degeneration) is an autosomal recessive defect in ATP7B resulting in copper storage disease. It affects primarily liver, CNS, and cornea of eye **[78]**.
- Menkes Kinky Hair Syndrome is an X-linked neurodegenerative disease associated with mutation in ATP7A transporter resulting in low

serum copper levels and symptoms of copper deficiency including brittle, kinky hair and death by year 3 of life is usual **[79]**.

Cobalt (Co):

Cobalt forms an integral part of vitamin B12 and is required as a constituent of this vitamin **[80]**.

1.4.3.5.1. Functions of Cobalt:

In formation of cobamide coenzyme (Adenosyl co-enzyme), cobalt of B12 undergoes successive reduction in a series of steps catalysed by the enzyme "B12 reductase", which requires NADH and FAD **[81]**. Cobalt is required to maintain normal bone marrow function and required for development **[82]** and maturation of red blood cells **[83]**. A deficiency of cobalt results in decreased B12 supply which produces nutritional macrocytic anaemia **[84]**. Excess of cobalt results in overproduction of red blood cells causing polycythaemia. The polycythaemic effect may be due to inhibition of certain respiratory enzymes, e.g. cytochrome oxidase, succinate dehydrogenase, etc. leading to relative anoxia **[85]**. Cobalt may act as a cofactor for enzyme like glycylglycine dipeptidase of intestinal juice **[86]**.

1.4.3.5.2. Dietary sources of Cobalt:

Main sources are foods from animal sources **[87]**.

1.4.3.5.3. Absorption, Transport, Metabolism, and Excretion of Cobalt:

About 70 to 80 percent of the dietary cobalt is absorbed readily from the intestine. Isotopic studies have shown that about 65 per cent of the ingested cobalt is excreted almost completely through the kidney. Cobalt is stored mainly in the liver which is the principal storage site and only trace amount present in other tissues **[42]**.

1.4.3.5.4. Clinical Significance of Cobalt:

Cobalt deficiency results in anorexia, fatty liver, macrocytic anaemia, wasting and haemosiderosis of spleen **[88]**. Toxicity is rare and has been described in dialysis patients and heavy drinkers of beer that is contaminated with cobalt, leading to cardiomyopathy.

1.4.3.6. Chromium (Cr):

Chromium is an insulin cofactor. Chromium deficiency can occur in long-term parenteral nutrition, leading to glucose intolerance and neuropathy **[89]**.

1.4.3.6.1. Functions of Chromium:

The main biological role of chromium in man appears to potentiate the action of insulin, as part of a low molecular weight chromium binding substance (chromodulin) **[90]**. Chromium may also be important in gene expression, lipoprotein metabolism and in maintaining nucleic acid structure **[91]**.

1.4.3.6.2. Dietary Sources of Chromium:

Dietary sources of chromium include yeast, meat, whole grains, mushrooms and nuts **[75]**.

1.4.3.6.3. Absorption, Transport, Metabolism, and Excretion of Chromium:

Chromium is absorbed poorly in the diet. It is absorbed mainly in the small intestine by a pathway it appears to share with zinc **[42]**. Hexavalent chromium is better absorbed and much more toxic than trivalent chromium **[92]**. Both transferrin and albumin are involved in chromium absorption and transport **[93]**. Transferrin binds the newly absorbed chromium at site B, while albumin acts as an acceptor and transporter of

chromium if the transferrin sites are saturated **[94]**. Other plasma proteins, including β- and γ-globulins and lipoproteins, bind chromium **[95]**.

1.4.3.6.4. Clinical Significance of Chromium:

Impaired Glucose Tolerance and Diabetes: A lot of adults are thought to have impaired glucose tolerance and it has been suggested that poor chromium nutritional status may be a factor. However, the variability of dietary chromium intake and the lack of an easily usable laboratory or clinical marker to identify those patients with poor chromium status create difficulties. Chromium therapy in the control and prevention of diabetes is of considerable interest and the subject of much controversy **[96]**.

Cardiovascular Disease: Chromium depletion has long been thought to be associated with an increased cardiovascular risk. It has been found that chromium supplementation increase HDL cholesterol and decrease insulin levels **[97]**.

1.4.3.7. Selenium (Se):

1.4.3.7.1. Functions of Selenium:

Glutathione peroxidase is a selenium dependent enzyme. The enzyme has a role in oxidative damage by free radicals. The enzyme is critically important for the membrane stability of RBCs. Selenium has sparing action on vitamin E, by three ways. It promotes digestion, absorption of lipids and vitamin E. It is a part of glutathione peroxidase, prevents peroxidation of poly unsaturated fatty acids in the membranes. This in turn reduces the requirement of vitamin E. It helps in the retention of vitamin E in the blood. It is a cofactor for an enzyme involved in the synthesis of thyroid hormone **[98]**.

1.4.3.7.2. Dietary Sources of Selenium:

Selenium is rich in liver, kidney, finger nails. Usually plant products are good sources than animal based diet **[99]**.

1.4.3.7.3. Absorption, Transport, Metabolism, and Excretion of Selenium:

Selenium is absorbed from upper segment of the small intestine. The absorption is increased with deficiency **[100]**. Status is measured by measuring selenium or glutathione peroxidase in plasma, platelets, and RBC's or selenium levels in whole blood or urine. RBC selenium is an indicator of long-term status **[101]**. Selenium is stored in the body as selenocysteine in selenoproteins **[102]**. It is excreted in urine, feces and in breath as dimethyl selenide with a garlic-like odor **[103]**.

1.4.3.7.4. Clinical Significance of Selenium:

1.4.3.7.4.1. Deficiency of Selenium

A range of deficiency states has been identified in humans **[35]**.

1.4.3.7.4.2. Severe Deficiency of Selenium

Keshan Disease: It is strongly supported by nutritional, biological, geological and chemical (selenium deficiency) factors **[104]**.

Kashin-Beck Disease. A type of severe arthritis is described in parts of China and neighboring areas of Russia where soil selenium is particularly low **[105]**.

1.4.3.7.4.3. Toxicity of Selenium

Areas of China and the United States have high amounts of selenium in soil, and locally produced food contains excess selenium. Clinical signs of selenosis are garlic odor in the breath, hair loss, and nail damage. The tolerable upper limit has been set at 400 μg/day for adults and less for children **[35]**.

1.4.3.8. Molybdenum (Mo):

Molybdenum (Mo) is a hard, silvery white metal occurring naturally as molybdenite, wulfenite, and powelite. Most molybdenum is used for the

production of alloys, as well as catalysts, corrosion inhibitors, flame retardants, smoke depressants, lubricants, and molybdenum blue pigments. Molybdenum is an essential trace element with the importance of molybdenum-containing organic compounds in biological systems identified over 80 years ago **[106]**.

1.4.3.8.1. Functions of Molybdenum:

Xanthine oxidase and aldehyde oxidase contain molybdoprotein, a substituted pterin to which molybdenum is bound by two sulfur atoms. Molybdenum deficiency causes depression of xanthine oxidase activity, increased excretion of xanthine and decreased uric acid excretion **[34]**.

1.4.3.8.2. Dietary Sources of Molybdenum:

Legumes, grains and organ meats are good food sources of molybdenum; fruits, root and stem vegetables, and muscle meats are relatively poor ones **[107]**.

1.4.3.8.3. Absorption, Transport, Metabolism, and Excretion of Molybdenum:

Between 25% and 80% of ingested molybdenum is absorbed predominately in the stomach and small intestine, **[95]** with the majority of absorbed molybdenum retained in the liver, skeleton, and kidney. In blood, molybdenum is extensively bound to α2-macroglobulin and RBC membranes **[106]**.

Molybdenum can cross the placental barrier, and increased intake of molybdenum in the diet of the mother can increase its level in the liver of the neonate **[108]**.

1.4.3.8.4. Clinical Significance of Molybdenum:

Molybdenum is vital to human health through its inclusion in at least three enzymes: xanthine oxidase, aldehyde oxidase, and sulfite oxidase. The active site of these enzymes binds molybdenum in the form of a cofactor "molybdopterin" **[109]**. Dietary molybdenum deficiency is rare with a single case reported because of total parenteral nutrition in a man with Crohn's disease **[106]**. Molybdenum cofactor deficiency is a recessively inherited error of metabolism due to a lack of functional molybdopterin. The symptoms include seizures, anterior lens dislocation, decreased brain weight, and usually death prior to 1 year of age **[109]**. Molybdenum toxicity is rarely reported, as there are few known cases of human exposure to excess molybdenum. High dietary and occupational exposures to molybdenum have been linked to elevated uric acid in blood and an increased incidence of gout **[95]**. Molybdenum is rapidly eliminated in both urine and bile, with urine excretion predominating when intake is high **[106]**.

1.4.3.9. Manganese (Mn):

1.4.3.9.1. Functions of Manganese:

Manganese is a component of certain enzymes (e.g. pyruvate carboxylase, mitochondrial superoxide dismutase, arginase) and is also an activator of many others (e.g. hydrolases, glycosyl transferases, kinases, decarboxylases), so deficiency could potentially affect the metabolism of carbohydrates, glycosaminoglycans and cholesterol.

1.4.3.9.2. Dietary Sources of Manganese:

Good dietary sources of manganese include leafy vegetables, unrefined cereals and tea **[75]**.

1.4.3.9.3. Absorption, Transport, Metabolism, and Excretion of Manganese:

Roughly, 2% to 15% of dietary manganese is absorbed in the small intestine. Dietary factors that affect manganese absorption include iron, calcium, phosphates, and fiber **[95]**. Manganese absorption is age dependent, with infants retaining higher levels of manganese than adults do. Manganese is a normal component in tissue with the highest levels found in fat and bone. Though accumulation of manganese in the healthy population has not been observed, chronic liver disease or other types of liver dysfunction can reduce manganese elimination and promote accumulation in various regions of the brain **[110]**. Manganese elimination occurs predominately through the bile **[111]**.

1.4.3.9.4. Clinical Significance of Manganese:

Manganese is biochemically essential as a constituent of metalloenzymes and as an enzyme activator. Manganese containing enzymes include arginase, pyruvate carboxylase, and manganese superoxide dismutase in the mitochondria. Manganese-activated enzymes include hydrolases, kinases, decarboxylases, and transferases. Many of these activations are not specific to manganese and other metal ions (magnesium, iron, or copper) can replace manganese as an activator and mask the effects of manganese deficiency. Blood clotting defects, hypercholesterolemia, dermatitis, and elevated serum calcium, phosphorus, and alkaline phosphatase activity have been observed in some subjects who underwent experimental manganese depletion **[95]**. Low levels of manganese have been associated with epilepsy, **[109]** hip abnormalities, joint disease, congenital malformation, **[95]** heart and bone problems, and stunted growth in children **[111]**. Manganese toxicity causes nausea, vomiting, headache, disorientation, memory loss, anxiety, and compulsive laughing or crying. Chronic manganese toxicity resembles

Parkinson's disease with akinesia, rigidity, tremors, and mask-like faces **[109]**. A clinical condition named locura manganica (manganese madness) was described in Chilean manganese miners with acute manganese aerosol intoxication **[112]**.

1.5. Methods of Trace Elements Estimations:

Commonly used techniques for trace-element analysis in human biological material are flame atomic absorption spectrometry (FAAS), graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma atomic emission spectrometry (ICP-AES), and inductively coupled plasma mass spectrometry (ICP-MS) **[113]**.

1.6. Aim of the Study

To assess the role of trace and ultra-trace elements in the pathogenesis of pre-eclampsia.

CHAPTER TWO

Subjects, Materials, and Methods

2. MATERIALS & METHODS

2.1. Materials

2.1.1. Study Settings:

This study was carried out on in Babylon Teaching Hospital for Gynecology and Pediatrics, Babylon Province, Hilla City. The study samples patients attended the outpatient clinic, emergency room, labour room or in the obstetric wards. All samples were collected from November 2014 till February 2015. The laboratory workup for diagnosis was performed in the hospital laboratory. While the biochemical tests under study were performed at the laboratory of the Department of Clinical Biochemistry, College of Medicine, University of Babylon.

2.1.2. Study Design:

This is a case-control study.

2.1.3. Study Population:

It included 120 women, sixty of them were patients diagnosed with preeclampsia in the third trimester and the other sixty were healthy pregnant women (controls) in the third trimester.

2.1.4. Ethical Issues:

a- Approval by scientific committee of the Clinical Biochemistry Department, College of Medicine at University of Babylon, Iraq.

b- Approval by Babylon Health Directorate, Ministry of Health and Information Center for Research & Development of Babylon Province.

c- The objectives and methodology were explained to all of them and verbal consent participant in the study has been taken.

2.1.5. Data Collection:

Exclusion Criteria:

- Age over 40,
- BMI > 30,
- Previous history of pre-eclampsia,
- Family history of pre-eclampsia,
- Conditions in which the placenta is enlarged like multiple pregnancy and hydrops fetalis,
- Pre-existing hypertension or renal disease,
- Pre-existing vascular disease (as in diabetes or autoimmune vasculitis),
- Antiphospholipid Syndrome,
- Smoking **[3]**.

2.1.6. Study Instruments:

a- Questionnaire**:** included sociodemographic characteristics (name, age, occupation, educational level, residence, and gestational age), information about current pregnancy (symptomatology like oliguria, haematuria, right hypochondrial pain, presence of ecchymosis and bruises , any complications like intrauterine growth retardation, oligohydramnios, HELLP Syndrome, and eclamptic fit), past obstetrical, medical, social and family history.

b- Investigations**:** included assessment of proteinuria, haematological, biochemical and coagulation profile. Ultrasound and Doppler study of umbilical artery. All these were done to diagnose and assess the severity of PE.

c- Anthropometric measurement: Include: weight (Kg), height (m) and BMI**.**

Body Mass Index (BMI) was calculated by weight (in kilograms) divided by the square of height (in meters); weight and height were measured by the same scale for the whole sample subjects.

BMI = Weight (kg) / Square Height (m^2).

2.2. Blood Collection:

Venous blood samples were drawn from all subjects using 5 ml disposable syringe. Five milliliters of blood were obtained from each subject by venepuncture and pushed slowly into plain disposable tubes. Blood was allowed to clot at 37˚C for 10-15 minutes and then centrifuged at 2000 xg for approximately 10-15 minutes then the sera were divided into five aliquots and stored at -20˚C until analysis (the concentrations of serum iron (Fe), zinc (Zn), magnesium (Mg), copper (Cu), cobalt (Co), chromium (Cr), selenium (Se), molybdenum (Mo), and manganese (Mn) were measured.)

Urine samples were collected for measurement of urinary protein/creatinine ratio **[114]**.

2.3. Chemicals:

Chemicals and kits used in this study are listed in table (2-1):

Table (2-1): Chemicals used in the study

No.	Chemical Substance	Origin
1	Creatinine Kit	SYRBIO (Syria)
2	Total Protein Kit	SYRBIO (Syria)
3	Total Iron Kit	Spectrum (Egypt)
4	Zinc Kit	Spectrum (Egypt)
5	Magnesium Kit	Biolabo SA (France)
6	Standard Solution of Copper (1000 ppm)	Fluka (Switzerland)
7	Standard Solution of Cobalt (1000 ppm)	Fluka (Switzerland)
8	Standard Solution of Chromium (1000 ppm)	Fluka (Switzerland)
10	Standard Solution of Manganese (1000 ppm)	Fluka (Switzerland)
11	Standard Solution of Molybdenum (1000 ppm)	Fluka (Switzerland)
12	Standard Solution of Selenium (1000 ppm)	Fluka (Switzerland)
13	Nitric Acid (HNO_3) 2%	Fischer (Germany)

2.4. Instruments:

The instruments and tools used in this study are shown in table (2-2).

Table (2-2): Instruments and Tools.

No.	Instruments and Materials	Origin
1	Atomic Absorption Spectrophotometer	PG Instruments Ltd (UK)
2	Atomic Absorption Spectrophotometer	Shimadzu (Japan)
3	Spectrophotometer PD-303 UV	Cecil (England)
4	Centrifuge EBA 20	Hettich (Germany)
5	Incubator	Fisher scientific (USA)
6	Distillator	Bibby science (England)
7	Balance	Sartorius (Germany)
8	Micropipettes (5-50 µl)	Slamed (Germany)
9	Micropipette (100-1000 µl)	Slamed (Germany)
10	Deep Freeze	GFL / Germany
11	Volumetric Flask (25,50,100 mL)	China
12	Pipettes (1,2,5,10 mL)	China
13	Disposable syringes (5 mL)	Medical jet (Syria)
14	Disposable test tube (10 mL)	Meheco (China)
15	Eppindorf tube (1.5 mL)	China

2.5. Methods:

2.5.1. Determination of Urinary Creatinine Concentration.

Principle:

Creatinine, in alkaline picrate solution, forms a color complex. The rate of formation of complex is measured, the effect of bilirubin and glucose are reduced by using the kinetic procedure **[115]**.

Samples:

Urine was diluted 1/50 with distilled water.

Reagents

R_1	Sodium hydroxide	313 mmol/l
R_2	Picric Acid	35 mmol/l
R_3	Standard	2 mg/l

Reagents are stable at room temperature up to the expiry date.

Preparation of working reagent:

The reagent was prepared by mixing proportionally 1/1 the reagents R_1 and R_2. Stability: 1 month at 20º-25º C.

Procedure:

Wavelength	500 nm (480-520 nm)
Temperature	25 ºC
Cuvette	1 cm light path
Measurement	Against air or distilled water
Method	Kinetic–increasing

If the absorbance of the working reagent is higher than 0.4 at 492 nm the sample must be diluted.

	Standard	Sample
Standard	100 µl	-
Sample	-	100 µl
Working reagent	1 ml	1ml

Solutions have been mixed, and after 30 seconds, the absorbance has been read (A_1) and exactly 1 minute after first reading (A_2).

Calculation:

Concentration of creatinine in urine =

[($A_2 – A_1$) Sample) ÷ (($A_2 – A_1$) Standard)] X 100

100 = Standard concentration x dilution factor.

2.5.2. Determination of Urinary Total Protein Concentration:

Principle:

Proteins modify spectrum of absorption of the complex pyrogallol red molybdate. Globlins together with albumin react. The absorbance read at 598 nm which is proportional to the concentration in proteins.

Reagents:

1.R_1: Pyrogallol reagent

Pyrogallol red	0.06 mmol/l
Sodium molybdate	0.04 mmol/l
Succinic acid	50 mmol/l
Detergent	Q.S.

Quaternary Surfactant (Q.S.) is a powerful acid liquid detergent sanitizer. It is a blend of quaternary ammonium compound, acids, and nonionic surface active agents.

2. R_2: Standard 100 mg/dl – 1 g/l.

Preparation:

The reagent was ready for use and stable up to the date of expiration as specified. Contamination after opening must be avoided.

Procedure:

Wavelength	600 nm
Temperature	37º C (25º C – 30º C)
Cuvette	1 cm light path
Method	Endpoint–increasing

If the absorbance of the working reagent is higher than 0.25 at 600 nm the reagent cannot be used.

	Blank	Standard	Sample
Sample	-	-	50 µl
Standard	-	50 µl	-
Reagent	3 ml	3 ml	3 ml

Solutions have been mixed, and after 10 minutes incubation at room temperature absorbance (A) was read against the blank, the final color is stable for at least 30 minutes.

Calculation:

The conc. of protein (g/l) = [$(A_{Sample}) \div (A_{Standard})$] x Standard concentration (g/l)

2.5.3. Determination of Serum Iron Concentration:

Principle:

Serum iron was measured by colorimetric method. Iron reacts with chromazurol B (CAB) and cetyltrimethyl-ammonium bromide (CTMA) to form a colored ternary complex with an absorbance measured at 623 nm. The

intensity of the color produced, is directly proportional to the concentration of iron in the sample **[116]**.

Reagents:

Reagents	Composition
Standard Iron (ST)	200 µg/dl 35.8 µmol/l
Acetate buffer PH 4.7 **Chromazurol B** **Cetyltrimethyl-ammonium bromide**	50 mM 0.13 mM 0.82 mM
Preservatives and stabilizers	

Procedure:

	Reagent : Blank	Standard	Sample
Reagent (R)	1 ml	1 ml	1 ml
Standard	-	40 µl	-
Sample	-	-	40 µl

The tubes were mixed, and incubated for 5 minutes at 37 ºC. The absorbance of the standard and sample against reagent blank were read.

Calculation:

Iron (µg/dl) = [$(A_{Sample}) \div (A_{Standard})$] x 200 (Standard Concentration)

2.5.4. Determination of Serum Zinc Concentration:

Principle:

Serum zinc was estimated by spectrophotometric method. Zinc forms with 2-(5-Brom-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino)-phenol a red chelate complex. The increase in absorbance can be measured and is proportional to the concentration of total zinc in the sample **[117]**.

Reagents:

Reagents	Composition
Standard (St.)	200 µg/dl (30.6 µmol/l)
Reagent (R)	
5-Br-PAPS	0.02 mmol/l
Bicarbonate buffer pH 9.8	200 mmol/l
Sodium Citrate	170 mmol/l
Dimethylglyoxime	4 mmol/l
Detergent	1%

Procedure:

	Blank	Standard	Sample
Reagent (R)	1 ml	1 ml	1 ml
Standard (St)	-	50 µl	-
Sample	-	-	50 µl

The tubes were mixed, and then incubated for 5 min at 37 ºC. The absorbance of the sample (A_s) and the absorbance of standard (A_{st}) were measured against reagent blank.

Calculation:

Zinc (µg/dl) = [$(A_{specimen}) \div (A_{standard})$] x 200

2.5.5. Determination of Serum Magnesium Concentration:

Principle:

Serum magnesium was measured by Gindler, Heth and Khayam-Bashi method. Calmagite, a metallochromic indicator (1-[1-hydoxy-4-methyl-2-phenolazo]-2-naphtol-4-sulfonic acid), forms in basic buffered medium a colored complex with the magnesium. The absorbance, measured at 510-550

nm, is proportional to the concentration of magnesium in the specimen. Ethylene glycol tetra acetic acid (EGTA) reduces calcium interference, potassium cyanide (KCN) reduces interference of heavy metals and surfactants reduce the interference of proteins and lipemia **[118]**.

Reagents Preparation:

Reagents are ready for use.

Reagents:

	Composition
Vial R_1 Calmagite Reagent	Calmagite ≥ 100 µmol/l AMP ≥ 100 mmol/l KCN 6.14 mmol/l EGTA 250 µmol/l Surfactants
Vial R_2 Standard	Magnesium 2 mg/dl (0.822 mmol/l)

Procedure:

- Reagent and specimens were let stand at room temperature.
- A constant temperature was maintained as the reaction is temperature sensitive.
- Reaction is stable for 60 minutes.

Pipette into well identified test tubes	**Blank**	**Standard**	**Assay**
Reagent (R_1)	1 ml	1 ml	1 ml
Demineralized water	10 µl	-	-
Standard (R_2) (2 mg/dl)	-	10 µl	-
Specimen	-	-	10 µl

Calculation:

Magnesium conc (mg/dl) = [(Abs $_{(Assay)}$) ÷ (Abs $_{(Standard)}$)] X 2 mg/dl (Standard concentration)

Determination of Serum Cu, Co, Cr, Se, Mo, and Mn Concentration:

Graphite furnace atomic absorption spectrophotometer was used to determine the trace elements in serum samples**.**

Principle:

In the atomic absorption spectrometry techniques, the samples are vaporized into free, neutral atoms and illuminated by a light source that emits the atomic spectrum of the element under analysis. The absorbance gives a quantitative measure of the concentration of the element. Inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) are multi-element techniques. In ICP-AES the atoms of the sample are excited by, for example, argon plasma at very high temperatures. The emitted light is directed to a detector, and the optical signals are processed to values for the concentrations of the elements. In ICP-MS a mass spectrometer separates and detects ions produced by the ICP, according to their mass-to-charge ratio. Dilution of biological fluids is commonly needed to reduce the effect of the matrix. Digestion using acids and microwave energy in closed vessels at elevated pressure is often used. Matrix and spectral interferences may cause problems. Precautions should be taken against trace-element contamination during collection, storage and processing of samples. For clinical problems requiring the analysis of only one or a few elements, the use of FAAS may be sufficient, unless the higher sensitivity of GFAAS is required. For screening of multiple elements, however, the ICP techniques are preferable **[113]**.

2.5.6. Determination of Serum Copper Concentration:

Sample Digestion:

One milliliter of serum was transferred to a Teflon beaker and 10 ml of concentrated nitric acid and 2.5 ml concentrate perchloric acid were added. The sample was then brought very slowly to boiling on a hot plate and heated to dryness. If sample blackening occurred during the fuming stage, nitric acid was added dropwise, then the sample was cooled, dissolved again in distilled water and concentrated HCl (10:1) and brought to a volume of 25 ml in a volumetric flask. The solution was analyzed against calibration curves **[119]**.

Preparation of standard solutions and standard curve of copper from stock solutions (1000 Cu ppm):

Five different standard solutions of copper (0, 25, 50, 75,100) ppb were prepared from stock solution of (1000) ppm using dilution law ($N_1 V_1 = N_2 V_2$). Dilutions were carried out in stages and acidified by using nitric acid > 1% to avoid precipitation.

1. Preparation of 100 ppm : 5 ml of 1000 ppm copper solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppm X V_1 = 100 ppm X 50 ml

V_1= 5 ml

2. Preparation of 10 ppm : 5 ml of 100 ppm copper solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

100 ppm X V_1 = 10 ppm X 50 ml

V_1= 5 ml

3. Preparation of 1 ppm (1000 ppb) : 5 ml of 10 ppm copper solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

10 ppm X V_1 = 1 ppm X 50 ml

V_1= 5 ml

4. Preparation of 100 ppb : 5 ml of 1000 ppb copper solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 100 ppb X 50 ml

V_1= 5 ml

5. Preparation of 75 ppb : 3.75 ml of 1000 ppb copper solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 75 ppb X 50 ml

V_1= 3.75 ml

6. Preparation of 50 ppb : 2.5 ml of 1000 ppb copper solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 50 ppb X 50 ml

V_1= 2.5 ml

7. Preparation of 25 ppb : 1.25 ml of 1000 ppb copper solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 25 ppb X 50 ml

V_1= 1.25 ml

8. Preparation of 0 ppb : this means pure distilled water was used as standard solution.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 0 ppb X 50 ml

V_1= 0 ml

9. Preparation of standard curve of copper : It was prepared by addition of 10 µl of (0, 25, 50, 75, 100) ppb copper solution to the graphite tube of graphite

furnace atomic absorption instrument. Standard curve of copper was drawn by instrument after it measured the absorbencies of them.

10. Concentrations of serum copper in patients and control were measured by the same instrument dependence on the previous standard curve.

Condition of Copper Determination:

Table (2-3): Ideal conditions of copper determination.

Variable	Ideal condition
Wavelength	324.7 nm
Band width	0.4 nm
Lamp Current	3.0 mA
Integration Time	3.0 sec.
Sample Size	10 µl
Acidity	0.1% Nitric Acid

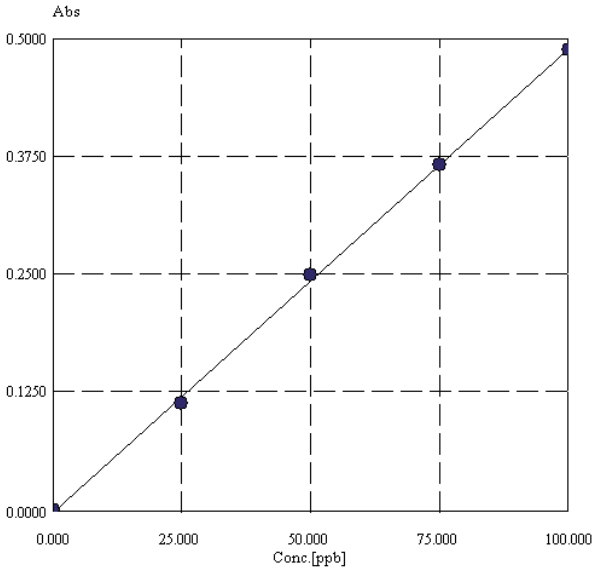

Fig (2.1): Standard curve of copper determination.

2.5.7. Determination of Serum Cobalt Concentration:

Sample Digestion:

One milliliter of serum was transferred to a Teflon beaker and 10 ml of concentrated nitric acid and 2.5 ml concentrate perchloric acid were added. The sample was then brought very slowly to boiling on a hot plate and heated to dryness. If sample blackening occurred during the fuming stage, nitric acid was added dropwise, then the sample was cooled, dissolved again in distilled water and concentrated HCl (10:1) and brought to a volume of 25 ml in a volumetric flask. The solution was analyzed against calibration curves **[119]**.

Preparation of standard solutions and standard curve of cobalt from stock solutions (1000 Co ppm):

Five different standard solutions of cobalt (0, 25, 50, 75,100) ppb were prepared from stock solution of (1000) ppm using dilution law ($N_1 V_1 = N_2 V_2$). Dilutions were carried out in stages and acidified by using nitric acid > 1% to avoid precipitation.

1. Preparation of 100 ppm : 5 ml of 1000 ppm cobalt solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppm X V_1 = 100 ppm X 50 ml

V_1= 5 ml

2. Preparation of 10 ppm : 5 ml of 100 ppm cobalt solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

100 ppm X V_1 = 10 ppm X 50 ml

V_1= 5 ml

3. Preparation of 1 ppm (1000 ppb) : 5 ml of 10 ppm cobalt solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

10 ppm X V_1 = 1 ppm X 50 ml

V_1= 5 ml

4. Preparation of 100 ppb : 5 ml of 1000 ppb cobalt solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 100 ppb X 50 ml

V_1= 5 ml

5. Preparation of 75 ppb : 3.75 ml of 1000 ppb cobalt solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 75 ppb X 50 ml

V_1= 3.75 ml

6. Preparation of 50 ppb : 2.5 ml of 1000 ppb cobalt solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 50 ppb X 50 ml

V_1= 2.5 ml

7. Preparation of 25 ppb : 1.25 ml of 1000 ppb cobalt solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 25 ppb X 50 ml

V_1= 1.25 ml

8. Preparation of 0 ppb : this means pure distilled water was used as standard solution.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 0 ppb X 50 ml

V_1= 0 ml

9. Preparation of standard curve of cobalt: It was prepared by addition of 10 µl of (0, 25, 50, 75, 100) ppb cobalt solution to the graphite tube of graphite

furnace atomic absorption instrument. Standard curve of cobalt was drawn by instrument after it measured the absorbencies of them.

10. Concentrations of serum cobalt in patients and control were measured by the same instrument dependence on the previous standard curve.

Condition of Cobalt Determination:

Table (2-4): Ideal conditions of cobalt determination.

Variable	Ideal condition
Wavelength	240.7 nm
Band width	0.2 nm
Lamp Current	7.0 mA
Integration Time	3.0 sec.
Sample Size	10 µl
Acidity	0.1% Nitric Acid

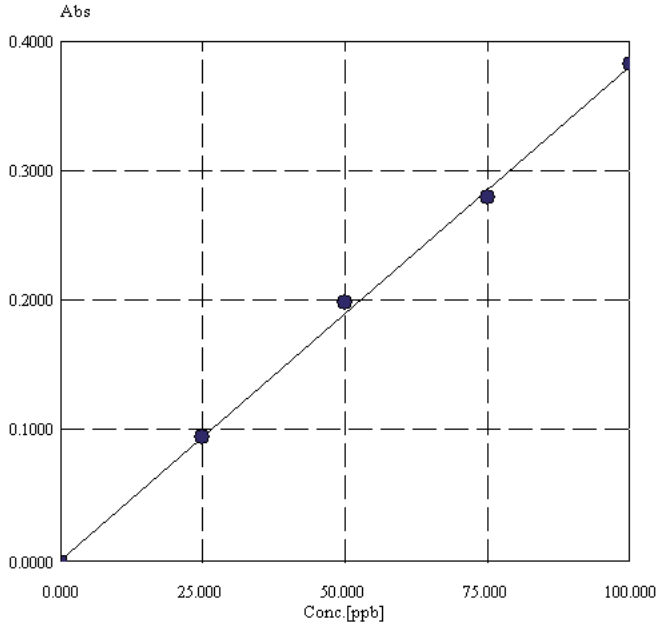

Fig (2.2): Standard curve of cobalt determination.

2.5.8. Determination of Serum Chromium Concentration:

Sample Digestion:

One milliliter of serum was transferred to a Teflon beaker and 10 ml of concentrated nitric acid and 2.5 ml concentrate perchloric acid were added. The sample was then brought very slowly to boiling on a hot plate and heated to dryness. If sample blackening occurred during the fuming stage, nitric acid was added dropwise, then the sample was cooled, dissolved again in distilled water and concentrated HCl (10:1) and brought to a volume of 25 ml in a volumetric flask. The solution was analyzed against calibration curves **[119]**.

Preparation of standard solutions and standard curve of chromium from stock solutions (1000 Cr ppm):

Five different standard solutions of chromium (0, 25, 50, 75,100) ppb were prepared from stock solution of (1000) ppm using dilution law ($N_1 V_1 = N_2 V_2$). Dilutions were carried out in stages and acidified using nitric acid > 1% to avoid precipitation.

1. Preparation of 100 ppm : 5 ml of 1000 ppm chromium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppm X V_1 = 100 ppm X 50 ml

V_1= 5 ml

2. Preparation of 10 ppm : 5 ml of 100 ppm chromium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

100 ppm X V_1 = 10 ppm X 50 ml

V_1= 5 ml

3. Preparation of 1 ppm (1000 ppb) : 5 ml of 10 ppm chromium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

10 ppm X V_1 = 1 ppm X 50 ml

V_1= 5 ml

4. Preparation of 100 ppb : 5 ml of 1000 ppb chromium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 100 ppb X 50 ml

V_1= 5 ml

5. Preparation of 75 ppb : 3.75 ml of 1000 ppb chromium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 75 ppb X 50 ml

V_1= 3.75 ml

6. Preparation of 50 ppb : 2.5 ml of 1000 ppb chromium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 50 ppb X 50 ml

V_1= 2.5 ml

7. Preparation of 25 ppb : 1.25 ml of 1000 ppb chromium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 25 ppb X 50 ml

V_1= 1.25 ml

8. Preparation of 0 ppb : this means pure distilled water was used as standard solution.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 0 ppb X 50 ml

V_1= 0 ml

9. Preparation of standard curve of chromium: It was prepared by addition of 10 µl of (0, 25, 50, 75, 100) ppb copper solution to the graphite tube of graphite furnace atomic absorption instrument. Standard curve of chromium was drawn by instrument after it measured the absorbencies of them.

10. Concentrations of serum chromium in patients and control were measured by the same instrument dependence on the previous standard curve.

Condition of Chromium Determination:

Table (2-5): Ideal conditions of chromium determination.

Variable	Ideal condition
Wavelength	357.9 nm
Band width	0.4 nm
Lamp Current	5.0 mA
Integration Time	3.0 sec.
Sample Size	10 µl
Acidity	0.1% Nitric Acid

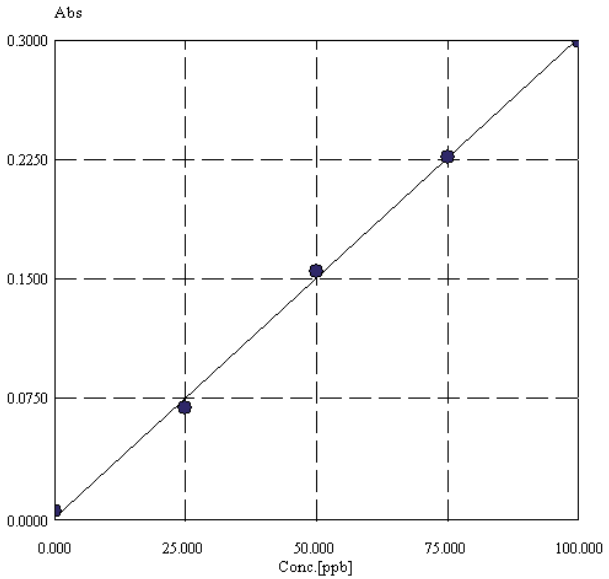

Fig (2.3): Standard curve of chromium determination.

2.5.9. Determination of Serum Selenium Concentration:

Sample Digestion:

One milliliter of serum was transferred to a Teflon beaker and 10 ml of concentrated nitric acid and 2.5 ml concentrate perchloric acid were added. The sample was then brought very slowly to boiling on a hot plate and heated to dryness. If sample blackening occurred during the fuming stage, nitric acid was added dropwise, then the sample was cooled, dissolved again in distilled water and concentrated HCl (10:1) and brought to a volume of 25 ml in a volumetric flask. The solution was analyzed against calibration curves**[119]**.

Preparation of standard solutions and standard curve of selenium from stock solutions (1000 Se ppm):

Five different standard solutions of selenium (0, 10, 20, 30, 40) ppb were prepared from stock solution of (1000) ppm using dilution law ($N_1 V_1 = N_2 V_2$). Dilutions were carried out in stages and acidified using nitric acid > 1% to avoid precipitation.

1. Preparation of 100 ppm : 5 ml of 1000 ppm selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppm X V_1 = 100 ppm X 50 ml

V_1= 5 ml

2. Preparation of 10 ppm : 5 ml of 100 ppm selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

100 ppm X V_1 = 10 ppm X 50 ml

V_1= 5 ml

3. Preparation of 1 ppm (1000 ppb) : 5 ml of 10 ppm selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

10 ppm X V_1 = 1 ppm X 50 ml

V_1= 5 ml

4. Preparation of 100 ppb : 5 ml of 1000 ppb selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 100 ppb X 50 ml

V_1= 5 ml

5. Preparation of 40 ppb : 20 ml of 1000 ppb selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 40 ppb X 50 ml

V_1= 20 ml

6. Preparation of 30 ppb : 15 ml of 1000 ppb selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 30 ppb X 50 ml

V_1= 15 ml

7. Preparation of 20 ppb : 10 ml of 1000 ppb selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 20 ppb X 50 ml

V_1= 10ml

8. Preparation of 10 ppb : 5 ml of 1000 ppb selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 10 ppb X 50 ml

V_1= 5 ml

9. Preparation of 0 ppb : this means pure distilled water was used as standard solution.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 0 ppb X 50 ml

V_1= 0 ml

10. Preparation of standard curve of selenium : It was prepared by addition of 10 µl of (0, 25, 50, 75, 100) ppb selenium solution to the graphite tube of graphite furnace atomic absorption instrument. Standard curve of selenium was drawn by instrument after it measured the absorbencies of them.

11. Concentrations of serum selenium in patients and control were measured by the same instrument dependence on the previous standard curve.

Condition of Selenium Determination:

Table (2-6): Ideal conditions of selenium determination.

Variable	Ideal condition
Wavelength	196.0 nm
Band width	0.4 nm
Lamp Current	5.0 mA
Integration Time	3.0 sec.
Sample Size	10 µl
Acidity	0.1% Nitric Acid

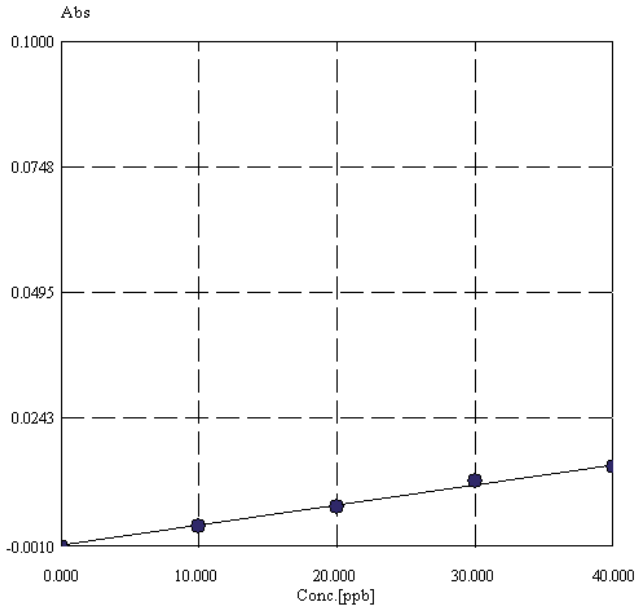

Fig (2.4): Standard curve of selenium determination.

2.5.10. Determination of Serum Molybdenum Concentration:

Sample Digestion:

One milliliter of serum was transferred to a Teflon beaker and 10 ml of concentrated nitric acid and 2.5 ml concentrate perchloric acid were added. The sample was then brought very slowly to boiling on a hot plate and heated to dryness. If sample blackening occurred during the fuming stage, nitric acid was added dropwise, then the sample was cooled, dissolved again in distilled water and concentrated HCl (10:1) and brought to a volume of 25 ml in a volumetric flask. The solution was analyzed against calibration curves **[119]**.

Preparation of standard solutions and standard curve of molybdenum from stock solutions (1000 Mo ppm):

Five different standard solutions of molybdenum (0, 100, 200, 300,400) ppb were prepared from stock solution of (1000) ppm using dilution law

($N_1 V_1 = N_2 V_2$). Dilutions were carried out in stages and acidified using nitric acid > 1% to avoid precipitation.

1. Preparation of 100 ppm : Five ml of 1000 ppm molybdenum solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppm X V_1 = 100 ppm X 50 ml

V_1= 5 ml

2. Preparation of 10 ppm : Five ml of 100 ppm molybdenum solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

100 ppm X V_1 = 10 ppm X 50 ml

V_1= 5 ml

3. Preparation of 1 ppm (1000 ppb) : Five ml of 10 ppm molybdenum solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

10 ppm X V_1 = 1 ppm X 50 ml

V_1= 5 ml

4. Preparation of 400 ppb : 20 ml of 1000 ppb molybdenum solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 400 ppb X 50 ml

V_1= 20 ml

5. Preparation of 300 ppb : 15 ml of 1000 ppb molybdenum solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 300 ppb X 50 ml

V_1= 15 ml

6. Preparation of 200 ppb : 10 ml of 1000 ppb molybdenum solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 200 ppb X 50 ml

V_1= 10 ml

7. Preparation of 100 ppb : Five ml of 1000 ppb molybdenum solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 100 ppb X 50 ml

V_1= 5 ml

8. Preparation of 0 ppb : this means pure distilled water was used as standard solution.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 0 ppb X 50 ml

V_1= 0 ml

10. Preparation of standard curve of molybdenum: It was prepared by addition of 10 µl of (0, 25, 50, 75, 100) ppb molybdenum solution to the graphite tube of graphite furnace atomic absorption instrument. Standard curve of molybdenum was drawn by instrument after it measured the absorbencies of them.

11. Concentrations of serum molybdenum in patients and control were measured by the same instrument dependence on the previous standard curve.

Condition of Molybdenum Determination:

Table (2-7): Ideal conditions of molybdenum determination.

Variable	Ideal condition
Wavelength	313.3 nm
Band width	0.4 nm
Lamp Current	5.0 mA
Integration Time	3.0 sec.
Sample Size	10 µl
Acidity	0.1% Nitric Acid

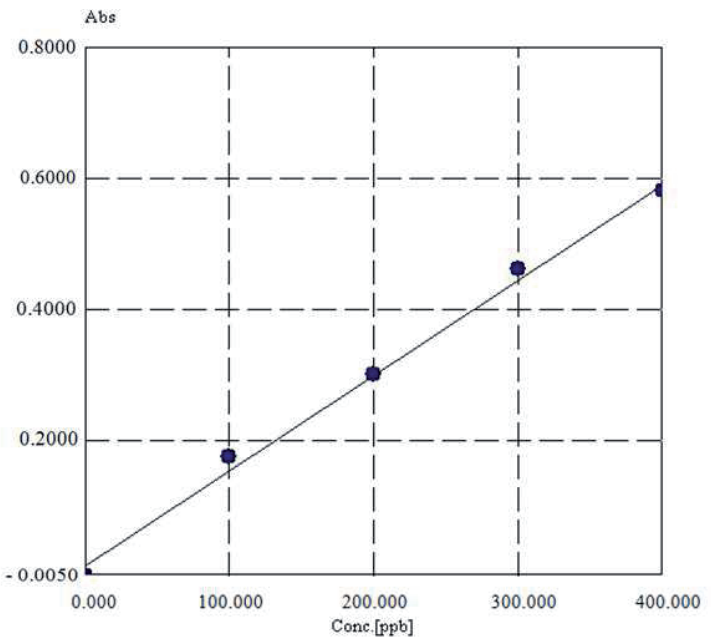

Fig (2.5): Standard curve of molybdenum determination.

2.5.11. Determination of Serum Manganese Concentration:

Sample Digestion:

One milliliter of serum was transferred to a Teflon beaker and 10 ml of concentrated nitric acid and 2.5 ml concentrate perchloric acid were added. The sample was then brought very slowly to boiling on a hot plate and heated to dryness. If sample blackening occurred during the fuming stage, nitric acid was added dropwise, then the sample was cooled, dissolved again in distilled water and concentrated HCl (10:1) and brought to a volume of 25 ml in a volumetric flask. The solution was analyzed against calibration curves**[119]**.

Preparation of standard solutions and standard curve of manganese from stock solutions (1000 Cu ppm):

Four different standard solutions of manganese (5, 10, 15, 20) ppb were prepared from stock solution of (1000) ppm using dilution law ($N_1 V_1 = N_2 V_2$). Dilutions were carried out in stages and acidified using nitric acid > 1% to avoid precipitation.

1. Preparation of 100 ppm : Five ml of 1000 ppm selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppm X V_1 = 100 ppm X 50 ml

V_1= 5 ml

2. Preparation of 10 ppm : Five ml of 100 ppm selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

100 ppm X V_1 = 10 ppm X 50 ml

V_1= 5 ml

3. Preparation of 1 ppm (1000 ppb) : Five ml of 10 ppm selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

10 ppm X V_1 = 1 ppm X 50 ml

V_1= 5 ml

4. Preparation of 100 ppb : Five ml of 1000 ppb selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 100 ppb X 50 ml

V_1= 5 ml

5. Preparation of 20 ppb : 10 ml of 1000 ppb selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 20 ppb X 50 ml

V_1= 10 ml

6. Preparation of 15 ppb : 7.5 ml of 1000 ppb selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 15 ppb X 50 ml

V_1= 7.5 ml

7. Preparation of 10 ppb : 5 ml of 1000 ppb selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 10 ppb X 50 ml

V_1= 5 ml

8. Preparation of 5 ppb : 2.5 ml of 1000 ppb selenium solution was diluted by distilled water since the solution was completed to 50 ml.

$N_1 V_1 = N_2 V_2$

1000 ppb X V_1 = 5 ppb X 50 ml

V_1= 2.5 ml

9. Preparation of standard curve of selenium : It was prepared by addition of 10 µl of (0, 25, 50, 75, 100) ppb selenium solution to the graphite tube of

graphite furnace atomic absorption instrument. Standard curve of selenium was drawn by instrument after it measured the absorbencies of them.

10. Concentrations of serum selenium in patients and control were measured by the same instrument dependence on the previous standard curve.

Condition of Manganese Determination:

Table (2-8): Ideal conditions of manganese determination.

Variable	Ideal condition
Wavelength	279.5 nm
Band width	0.2 nm
Lamp Current	20 mA
Integration Time	3.0 sec.
Sample Size	10 µl
Acidity	0.1% Nitric Acid

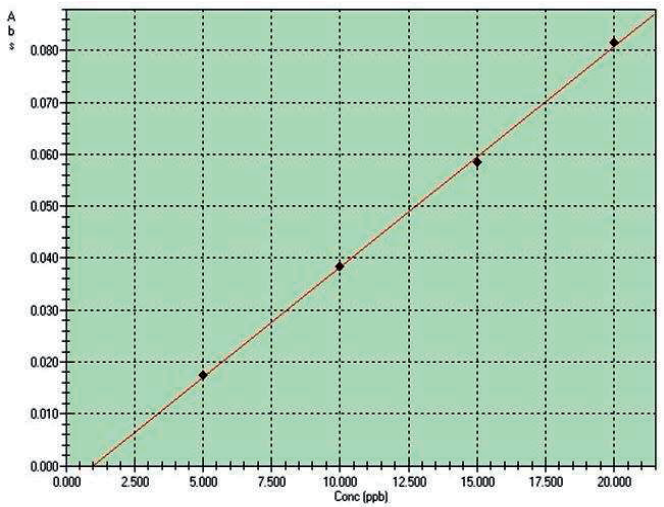

Fig (2.6): Standard curve of manganese determination.

2.6. Conversion of Parts Per Billion (ppb) to Microgram / Deciliter (µg/dl) and Milligram/Deciliter (mg/dl).

Parts per billion: Nanograms of solute per gram of solution; for aqueous solutions the units are often expressed as micrograms of solute per liter of solution (ppb) [133].

ppb = µg/liter = ng/mL

1 ppb = 1 µg/liter = 0.1 µg/dl

= 0.0001 mg/dl

2.7. Statistical Analysis

The results were expressed as mean ± standard error of mean. T-test and the linear regression analysis were used for determination of the level of significance. Statistical analyses were performed with Statistical Package for the Social Sciences (SPSS) version 21.0 software. A P value of < 0.05 was considered to be statistically significant.

CHAPTER THREE

Results and Discussion

3. Results and Discussion

3.1. Demographic Characteristics in Patients and Control.

Table 3.1. shows the characteristics of study sample:

Table 3.1 : Demographic characteristics of patients and control.

NO.	Characteristics	Control	Patient	P. Value
		Mean ± SEM	Mean ± SEM	
1	Maternal Age (Years)	26.85 ± 0.53	27.72 ± 0.66	> 0.05
2	Gestational Age(Weeks)	37.28± 0.28	36.93± 0.46	> 0.05
3	BMI (Kg/m^2)	27.83 ± 0.24	28.31 ± 0.20	> 0.05
4	Diastolic BP(mmHg)	77.60 ± 0.69	106.42 ± 2.03	< 0.05
5	Systolic BP(mmHg)	115.62 ± 0.90	159.63 ± 2.63	< 0.05
6	Protein/Creatinine Ratio	27.94 ± 0.19	38.82 ± 0.31	< 0.05
7	Parity			
	Parity Characteristics	Control		Patient	
		NO.	%	NO.	%
	0	4	6.7	31	51.7
	1-3	54	90	22	36.7
	≥ 4	2	3.3	7	11.7
8	Number of samples	60		60	

3.1.1. Age Distribution in patients and control:

There were no statistically significant difference between the mean age of patients (27.72 ± 0.66 years) and the mean age of control (26.85 ± 0.53 years), (P. value > 0.05) as shown in Table 3.1.

3.1.2. Distribution of Patients and Control Group by Gestational Age:

Table 3.1 showed the distribution of gestational age in patients and control. There were no statistically significant differences between means

of gestational ages for patients (36.93± 0.46 weeks) and control (37.28± 0.28 weeks), (P. value > 0.05).

3.1.3. Distribution of Patients and Control Group by Body Mass Index:

There were no statistically significant differences between the means of body mass index of patients (28.30 ± 0.20 Kg/m^2) and the mean of body mass index of control (27.83 ± 0.24Kg/m^2), (P. value > 0.05), as shown in Table 3.1.

3.1.4. Distribution of Patients and Control Group by Blood Pressure:

3.1.4.1. Distribution of Patients and Control by Diastolic Blood Pressure:

There were statistically significant differences between the mean of diastolic blood pressure of patients (106.42 ± 2.03 mmHg) and the mean of diastolic blood pressure of control (77.60 ± 0.69 mmHg), (P. value < 0.05), as shown in Table 3.1.

3.1.4.2 Distribution of Patients and Control Group by Systolic Blood Pressure:

Table 3.1demonstrated that there was statistically significant differences between the mean of systolic blood pressure of patients and the mean of systolic blood pressure of control (159.63 ± 2.63mmHg, 115.62 ± 0.90mmHg) respectively, (P. value < 0.05) .

3.1.5. Distribution of Patients and Control Group by Urinary Total Proteins/Creatinine Ratio:

Table 3.1 showed the distribution of urinary total proteins/creatinine ratio in both groups. There were statistically significant differences between mean of urinary total proteins/creatinine ratio for patients (38.82±0.31) and control (27.94±0.19), (P. value < 0.05).

The reliable detection of proteinuria is essential in differentiating those pregnancies with pre-eclampsia from those with gestational or chronic hypertension and, in the process, identifying those pregnancies most prone to adverse outcomes. The measurement of significant proteinuria, traditionally 300 mg excretion in a 24-hour period, is also prone to collection and measurement error. The collection of 24-hour urine samples is not practical as a routine test and so urine dipstick screening is employed as a first-line screening test with secondary tests employed to confirm positive dipstick diagnoses. Visual dipstick reading is unreliable but the use of automated dipstick readers significantly improves the accuracy of dipstick testing **[120]** and as such is recommended by The National Institute for Health and Care Excellence (NICE) for use in pregnancy.

NICE also recommends that quantification of proteinuria should follow diagnosis. There are two methods that NICE supports. The first is the 24-hour urine protein estimation and this requires that an assessment of sample completeness is undertaken, with measurement of creatinine excretion being the most common. NICE also supports the use of the protein/creatinine ratio test. This test is done on a 'spot' urine sample and is therefore much quicker. This test has been shown in numerous studies to be comparable to the 24-hour urine protein estimation. The threshold

for defining significant proteinuria by this test is 30 mg protein/mmol creatinine **[3]**.

3.1.6. Distribution of Patients and Control Group by Parity:

Table 3.1 revealed the deference in parity between patients and control. Fifty one percent of patients were primigravida while (6.7 %) were primigravida in the control group. This result can be explained by the fact that first pregnancy is regarded as a risk factor for pre-eclampsia.

3.2. Trace and Ultra Trace Elements Studies in Patients and Control.

3.2.1. Iron (Fe) Concentrations in Patients and Control.

Figure 3.6 showed that the mean serum iron level was significantly higher in patients than in control (186.498± 5.514 µg/dl vs. 94.392 ± 9.962 µg/dl), (P. value < 0.05).

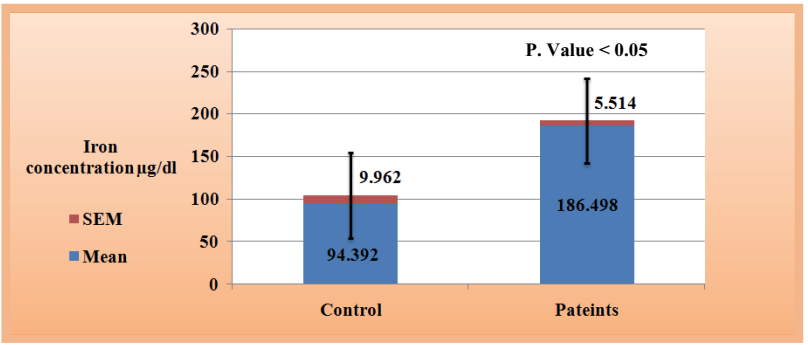

Figure (3.1): Serum iron concentrations (µg/dl) in patients and control (mean ±SEM).

An imbalance between pro-oxidants and anti-oxidants results in oxidative stress which increases the potential for the development of pre

eclampsia **[121]**. Free iron acts as pro-oxidant agent and it is released from ferritin by the reducing agents that convert Fe^{3+} into Fe^{2+}. Under stress or pathological conditions, it undergoes Fenton reaction and Haber – Weiss reaction to generate ROS, which in turn damage the biological macro molecules **[43]**. The elevated serum iron levels are due to hemolysis caused by physical destruction of red blood cells (RBCs) as a result of vasospam or abnormal endothelial cell erythrocyte interactions. Excess iron is a causative factor of oxidative stress (i.e., in its radical form) involved in the pathogenesis of pre eclampsia **[122]**. The excess iron released from destruction of RBCs can react with free radicals produced from cell membrane (as it is rich in polyunsaturated fatty acids) and circulating lipoproteins initiates lipid peroxidation **[123]**. In addition to this the damaged placenta is a site for release of free radicals (FR) in pre-eclampsia. The elevation or excess iron can also react with these released FR of placenta and can initiate and propagate lipid peroxidation both in placenta and systemic vasculature. This is one of the significant etiologic factors in the endothelial cell damage in pre-eclampsia **[124]**. The present study findings were agreed with Osman R, et al. **[125]** Hameed R, et al. **[126]**.

3.2.2. Zinc (Zn) Concentrations in Patients and Control.

Figure 3.11showed that the mean serum zinc level was significantly lower in patients than in control (57.283 ± 1.740 µg/dl vs. 87.535 ± 3.710 µg/dl), (P. value < 0.05).

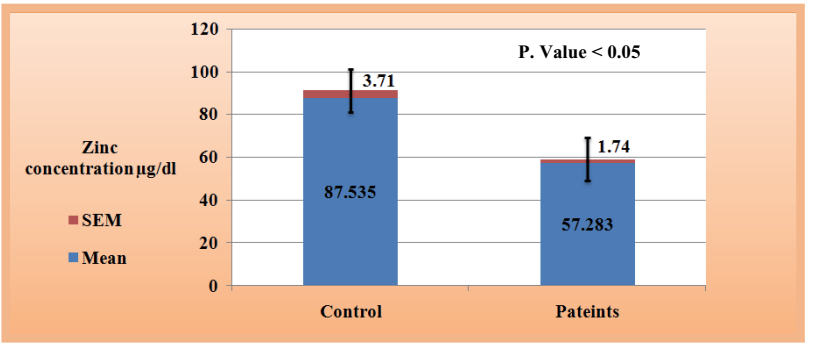

Figure (3.2) : Serum zinc concentrations (µg/dl) in patients and control (mean ±SEM).

Zn deficiency has been associated with complications of pregnancy part of which is pre-eclampsia. Zn is passively transferred from mother to fetus across the placenta and there is also decreased Zn binding capacity of maternal blood during pregnancy, which facilitates efficient transfer of Zn from mother to fetus. During pregnancy, there is a decline in circulating Zn and this increases as the pregnancy progresses possibly due to decrease in Zn binding and increased transfer of Zn from the mother to the fetus. Zn deficiency is also related to hemodilution and increased urinary excretion **[127]**. In pregnant women with preeclampsia, low serum zinc may be partly due to reduced concentrations of Zn-binding protein and estrogen caused by increased lipid peroxidation **[128,129]**. This leads us to hypothesize that zinc may play a role in pre-eclampsia through an increase of lipid peroxidation. The association between zinc and pre-eclampsia although attempts to modify the frequency of pre-eclampsia with zinc supplementation has not been successful. Recently, the role of oxidative stress or excessive lipid peroxidation has been implicated in pre-eclampsia. There is an imbalance between antioxidant enzyme activities and pro-oxidant production.

Maternal zinc deficiency is related with serum cortisol level that increases during normal pregnancy and it is much higher in preeclampsia. Ihan N, et al. have noted that women with preeclampsia as compared with normotensive pregnant women had lower zinc concentrations **[130]**. Zinc deficiency in the placental tissue might cause insufficiency of superoxide dismutase, an antioxidant enzyme. Furthermore, deficiency in placental zinc also plays a role in the biosynthesis of connective tissue, maintaining its integrity, which might have an impact on the structure of the spiral arteries **[131]**. Similar results were obtained by earlier studies by Jain S, et al. **[55]**, Mohammed K, et al. **[132]** Adeniyi A, et al. **[133]** Sarwar M, et al. **[134]**.

3.2.3. Magnesium (Mg) Concentrations in Patients and Control.

Figure 3.7 illustrated that the mean serum magnesium level was significantly lower in patients than in control (2.115 ± 0.077 mg/dl vs. 2.456 ± 0.092 mg/dl), (P. value < 0.05).

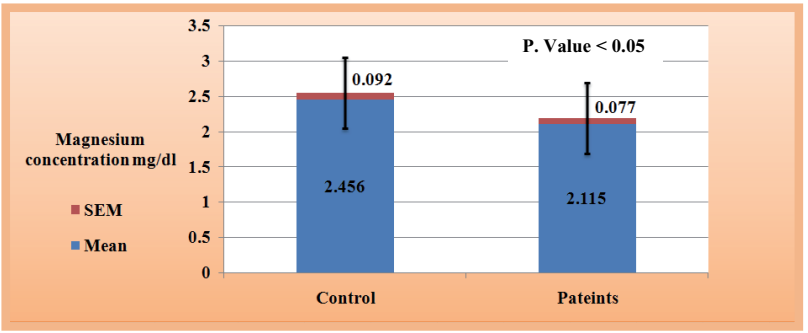

Figure (3.3): Serum magnesium concentrations (mg/dl) in patients and control (mean ±SEM).

The role of magnesium in obstetrics and its relationship to both foetal and maternal wellbeing has been established. The low concentration of magnesium in serum exposes the subject to a risk of pregnancy complications which includes pre-eclampsia. This is usually due to a defect in an enzymatic process which occurs as a result of low circulating magnesium to function as a co-factor. In this study, it has been found a decrease in serum magnesium in pregnant women with pre-eclampsia. This is consistent with the view of Lu and Nightingale who reported a reduced mortality rate from 16% to 8% in Bangladesh as a result of the introduction of a low dose of magnesium sulphate in pre-eclampsia patients **[135]**. The findings of this study were agree with Jain S, et al. **[55]** Nourmohammadi I, et al. **[136]**.

Magnesium is very important for cellular metabolism such as muscle contractility, secretions, neuronal activity, as well as cellular death. In the present study, low dietary intake and accelerated metabolism might be other contributing factors in hypomagnesaemia. This is suggestive of some role of this element in the rise of blood pressure. Although, calcium (Ca) alone might play a role in the rise of blood pressure, a proper balance of Ca and Mg is of vital importance to control blood pressure because blood vessels need Ca to contract, but they also require sufficient Mg to relax and open up **[137]**.

During pregnancy, there is a progressive decline in concentration of Mg in maternal serum possibly due to hemodilution, increased urinary excretion, and increased transfer of these minerals from the mother to the growing fetus **[138]**.

Hypoproteinaemia is another contributing factor since extracellular magnesium accounts for about 1% of the total body magnesium content.

About 55% of magnesium is free, 30% is associated with proteins (primarily albumin), and 15% is complexed with phosphate, citrate, and other anions **[139]**.

3.2.4. Copper (Cu) Concentrations in Patients and Control.

Figure 3.1 showed that the mean serum copper level was significantly lower in patients than in control (143.153 ± 3.316 µg/dl vs. 209.657± 8.679 µg/dl), (P. value < 0.05).

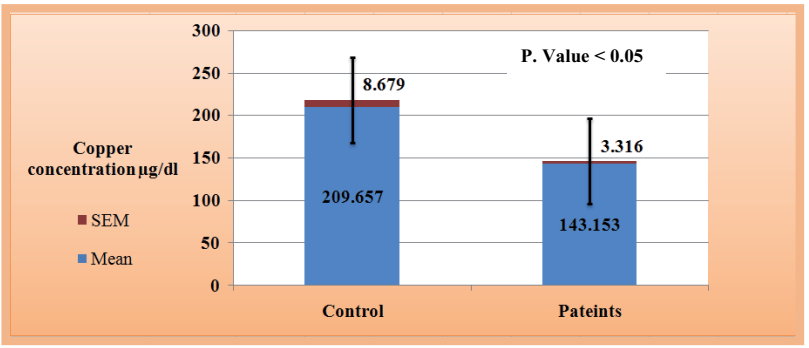

Figure (3.4): Serum copper concentrations (µg/dl) in patients and control (mean ±SEM).

Copper has been shown to be involved in the function of several cuproenzymes that are essential for life. It is known to be a cofactor of the antioxidant enzyme superoxide dismutase **[140]**. Gurer et al. **[141]** and Açikgoz et al. **[131]** reported increased malondialdehyde and decreased ceruloplasmin activity in the plasma of preeclamptic women. Based on these observations, low levels of copper in preeclamptic women may be associated with impairment of the cell antioxidant capacity and oxidant/antioxidant balance. Ceruloplasmin containing copper which catalyses the conversion of ferric ion to its ferrous form and favors the

absorption of iron from the gastro-intestinal tract. It also plays a role in the mobilization of iron to plasma from the tissue stores. Findings of present study indicated a decrease in the serum concentrations of copper in preeclamptic patients when compared to control subjects. Previous study have shown that copper deficiency is related more to fetal complications like birth defect than to maternal complication **[73]**.

3.2.5. Cobalt (Co) Concentrations in Patients and Control.

Figure 3.4 illustrated that the mean serum cobalt level was significantly lower in patients than in control (0.143 ± 0.007 µg/dl vs. 0.330 ± 0.007µg/dl), (P. value < 0.05).

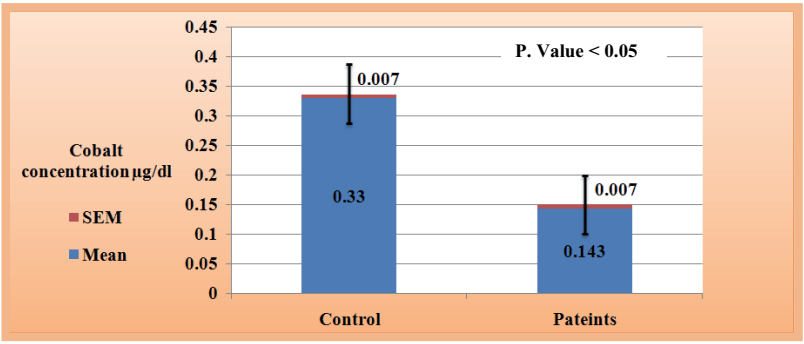

Figure (3.5): Serum cobalt concentrations (µg/dl) in patients and control (mean ±SEM).

Figure 3.5 demonstrated that there was a significant negative correlation between serum cobalt concentration and serum iron concentration in both patients and control groups (P. value < 0.05).

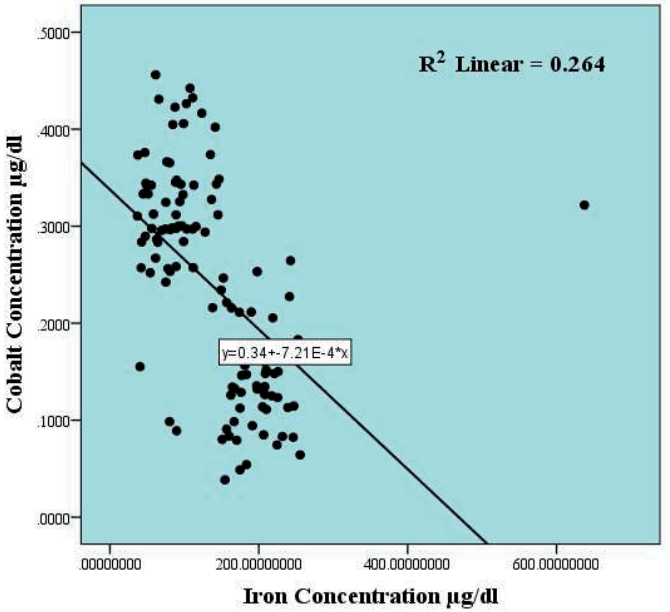

Figure (3.6): The relationship between iron level and cobalt concentrations in the sera of pre-eclamptic and normotensive pregnants.

In present study the heavy metal Co was decreased in PE; the level was similar to that reported in a previous study by Vigeh et at. **[141]**.

Cobalt is absorbed in the same pathway of iron. So any alteration in iron absorption will affect that of cobalt. Iron is mainly absorbed in the duodenum and upper jejunum. A transporter protein called divalent metal transporter 1 (DMT1) facilitates transfer of iron across the intestinal epithelial cells. DMT1 also facilitates uptake of other trace metals (manganese, copper, cobalt, and zinc). Iron within the enterocyte is released via ferroportin into the bloodstream. Iron is then bound in the bloodstream by the transport glycoprotein named transferrin **[142]**.

Normally, about 20 to 45% of transferrin binding sites are saturated with iron. About 0.1% of total body iron is circulating in bound form to transferrin. As the trace elements cobalt and manganese are absorbed and transported via the same mechanisms as iron, there might be a competition on active site of binding protein between cobalt and iron. And when there is iron overload, cobalt might be decreased **[143]**.

3.2.6. Chromium (Cr) Concentrations in Patients and Control.

Figure 3.2 illustrated that the mean serum chromium level was significantly lower in patients than in control (0.382 ± 0.0172 µg/dl vs. 0.678± 0.025 µg/dl), (P. value < 0.05).

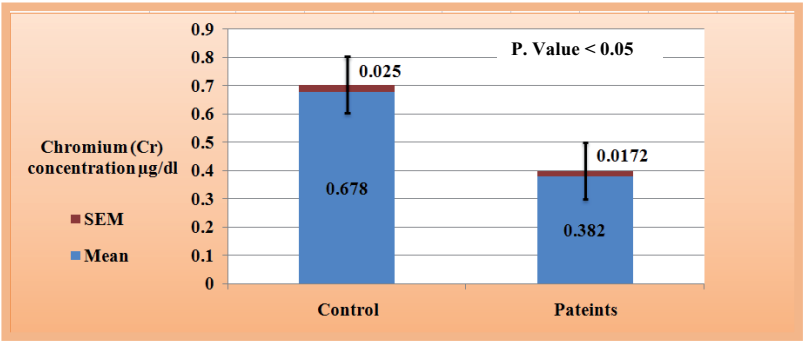

Figure (3.7): Serum chromium concentrations (µg/dl) in patients and control (mean ±SEM).

In the present study there was a significant negative correlation between serum chromium concentrations and serum iron concentrations in both patients and control groups (P. value < 0.05) as shown in the Figure 3.3.

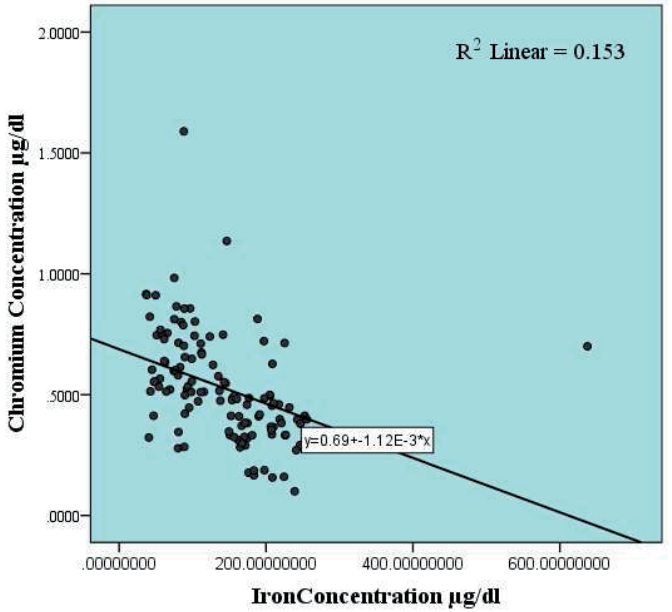

Figure (3.8): The relationship between iron level and chromium concentrations in the sera of pre-eclamptic and normotensive pregnants.

To understand the causes of chromium deficiency in subjects with PE, an interaction between chromium and iron metabolism should be explained. There are relatively few papers on the effect of Cr supplementation on the metabolism of other mineral substances. The relation between Cr and Fe has been investigated most since both these minerals are transported as transferrin-bound. At low Fe saturation, Cr and Fe bind preferentially to different binding sites. When, however, the Fe concentration is high, the two minerals compete for the same binding sites. This seems to be the reason why a lower Cr retention has been identified in patients suffering from hemochromatosis than in healthy subjects or patients with Fe deficiency **[144]**.

Evidence that Cr may impair Fe metabolism has been published by Ani M. et al. **[145]**. Fe homeostasis alteration has been reported by other authors too, the most significant alteration being detected in association with Cr-picolinate supplementation **[146]**. Alteration of Fe metabolism in association with Cr supplementation has also been reported by Anderson R. et al. **[147]**, decreased tissue Fe concentrations were detected in response to Cr supplementation.

Increasing of cortisol secretion is another factor that might contributes to decrease level of chromium. A number of studies confirm the association between Cr and the metabolism during increased physiological, pathological and nutritional stress. Cr demand in humans and animals increases during periods of higher stress – e.g., fatigue, trauma, gestation and different forms of nutritional (high-carbohydrate diet), metabolic, physical, and emotional stress as well as environmental effects. Under stress, secretion of the cortisol increases, acting as an insulin antagonist through increasing blood glucose concentration and reduction of glucose utilization by peripheral tissues. Increased blood glucose levels stimulate the mobilization of the Cr reserve, Cr being then irreversibly excreted in urine. Cr excretion in urine is enhanced by all stress-inducing factors **[144]**.

3.2.7. Selenium (Se) Concentrations in Patients and Control.

Figure 3.10 illustrated that the mean serum selenium level was significantly lower in patients than in control (2.546 ± 0.068µg/dl vs. 4.306 ± 0.050µg/dl), (P. value <0.05).

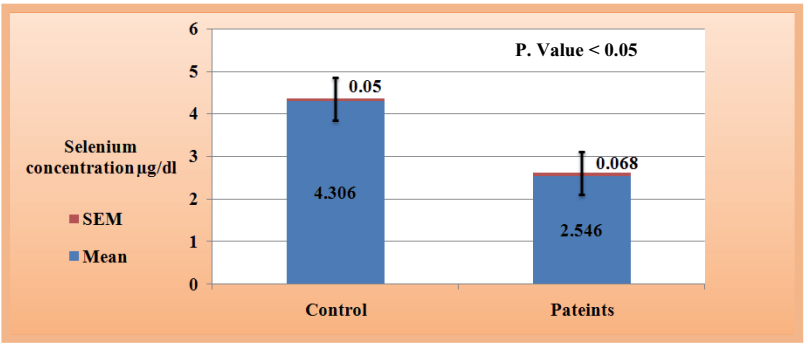

Figure (3.9): Serum selenium concentrations (µg/dl) in patients and control (mean ±SEM).

Selenium is known to behave as an antioxidant and peroxynitrite scavenger when incorporated into selenoproteins. It is the main element in glutathione peroxidase, an active enzyme in oxidative stress that reduces the formation of free radicals and peroxidation of lipoproteins. The low concentration of selenium in serum exposes the subject to an accumulation of free radicals and its associated pathogenesis of disease such as preeclampsia. On the other hand, low concentration of this element in blood might be an indication of active production of free radical and increased scavenging activity of either selenium or glutathione peroxidase. This study suggests that pre-eclampsia is associated with oxidative stress. This study agrees with the view of Witzum **[148]** who reported that increased oxidative stress as a result of peroxidation of low density lipoproteins is a common phenomenon in pre-eclampsia. Supplementation with this trace element may help reduce the pathogenesis of this disease and its complications **[149]**. Results of this study were also agreement with view of Rayman M et al **[150]** Mistry H. et al. **[151]** .

3.2.8. Molybdenum (Mo) Concentrations in Patients and Control.

There was no statistically significant difference between the mean of serum molybdenum concentrations of patients (2.304 ± 0.173 µg/dl) and the mean of serum molybdenum concentrations of control (2.670 ± 0.172 µg/dl), (P. value > 0.05) as described in Figure 3.9.

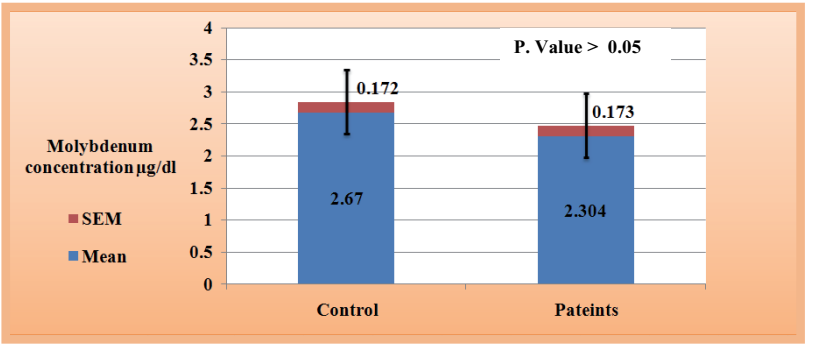

Figure (3.10): Serum molybdenum concentrations (µg/dl) in patients and control (mean ±SEM).

3.2.9. Manganese (Mn) Concentrations in Patients and Control.

Figure 3.8 showed that the mean serum manganese level was significantly lower in patients than in control (7.617 ± 0.293µg/dl vs. 10.847 ± 0.356µg/dl), (P. value < 0.05).

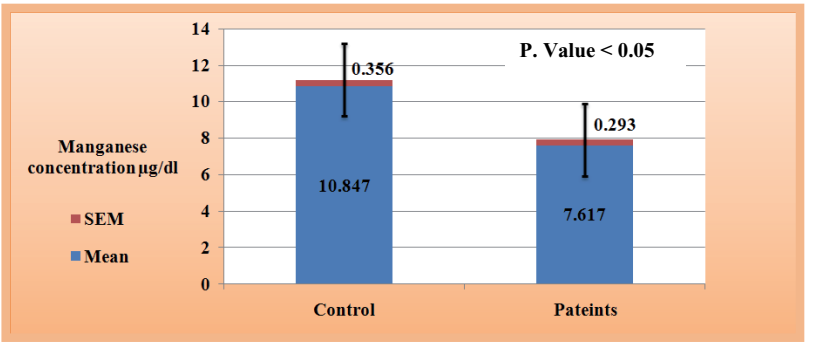

Figure (3.11): Serum manganese concentrations (µg/dl) in patients and control (mean ±SEM).

Several studies reported that low level of manganese in serum may cause accumulation of superoxides which could consequently trigger preeclampsia and its complications Hofmeyr G, et al **[152]** Lou G, et al. **[153]**. Results of this study agree with earlier findings by Mohammed K, et al. **[132]** Ohad K, et al. **[154]**.

Another mechanism by which manganese deficiency is involved in the pathogenesis of pre-eclampsia is through impairment of endothelial function. Manganese is an active element with arginine which is the precursor of the key determinant of endothelial function, nitric oxide, due to its function in the increase of smooth muscle relaxation, endothelial cell proliferation, decrease in endothelin-1-release, leukocyte adhesion, platelet aggregation, superoxide production, expression of monocytes chemotactic peptides, proliferation of smooth muscle cells and endothelial cell apoptosis **[153]**. Thus, reduction in serum manganese concentration in the blood of pre-eclamptic pregnant women as reported in the current study may be a cause rather than a resultant effect.

Conclusions

Alterations in the levels of serum trace and ultra-trace elements could contribute to the pathogenesis of PE.

Recommendations

- Further studies are recommended on supplementation (copper, chromium, cobalt, magnesium, manganese, selenium, and zinc) as a preventive method in patients at a risk of pre-eclampsia.
- Avoidance of iron supplementation in pregnant women at a risk of pre-eclampsia.

CHAPTER FOUR

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