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Correlation Between Heavy Metals and Neonatal Hyperbilirubinemia Among the Patients Attended at Bangabandhu Sheikh Mujib Medical University

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CORRELATION BETWEEN HEAVY METALS AND NEONATAL HYPERBILIRUBINEMIA AMONG THE PATIENTS ATTENDED AT BANGABANDHU SHEIKH MUJIB MEDICAL UNIVERSITY

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CERTIFICATE

It is a pleasure for us to certify that this thesis entitled **'Correlation between heavy metals and neonatal hyperbilirubinemia among the patients attended at 'Bangabandhu Sheikh Mujib Medical University'** is prepared by Mohammad Anwar Hossain, a doctoral fellow in Medical Science (Laboratory Medicine) of session 2015−16 at the Institute of Biological Sciences, University of Rajshahi, Bangladesh. He prepared the thesis under our joint supervision. This is his original work. The thesis is recommended and forwarded to the University of Rajshahi for necessary formalities leading to its acceptance in partial fulfillment of the requirements for the doctoral degree.

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I, the undersigned, have prepared this thesis entitled **'Correlation between heavy metals and neonatal hyperbilirubinemia among the patients attended at Bangabandhu Sheikh Mujib Medical University'**. This is an original work by me. This is herewith submitted to the Institute of Biological Sciences, University of Rajshahi, Bangladesh for necessary formalities leading to Doctor of Philosophy in Medical Science (Laboratory Medicine). No part of this work, in any form, has been submitted to any other academic institute for academic award.

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Dedicated to the Almighty Allah then My Parents and Family members

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LIST OF ABBREVIATIONS AND/OR ACRONYMS

Abbreviation	**Full Meaning**
HIC	High Income Countries
LMICs	Low and Mid Income Countries
NNJ	Neonatal jaundice
IUGR	Intrauterine growth retardation
LBW	Low birth weight
SDGs	Sustainable development goals
RBC	Red blood cells
BIND	Bilirubin-induced neurologic dysfunction
CMV	Cytomegalovirus
G6PD	Glucose-6-phosphate dehydrogenase
AAP	American Academy of Pediatrics
ATP	Adenosine triphosphate
FNB	Food and Nutrition Board
TNF	Tumor necrosis factor
BSMMU	Bangabandhu Sheikh Mujib Medical University
IBSc	Institute of Biological Sciences
SPSS	Statistical package for the social sciences
Fig.	Figure
gm	Gram
gm/dL	Gram per decilitre
i.e.	That is
kg	Kilogram
Ltd	Limited
µl	Microliter
mmol/l	Millimoles per liter
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mm^3	Cubic millimeter

nm	Nanometer
NS	Not significant
p	Probability
ppm	Parts per million
RBC	Red blood cell
rpm	Rotation per minute
S	Significant
SEM	Standard error of mean
SPSS	Statistical Package for Social Science
U/L	Unit per liter
viz.	Namely
WHO	World Health Organization
ANC	Antenatal care
Mg	Magnesium
Cu	Copper
Zn	Zinc
QC	Quality control
TB	Total Bilirubin
TSB	Total Serum Bilirubin
WBC	White Blood cell
CBC	Complete Blood Count
CNS	Central Nervous System
Nacl	Sodium Chloride
IMg	Ionized Magnesium
RS	Rotor syndrome
SD	Standard deviation
IEC	Institutional ethical committees
MTB	Methylthymol blue
ANOVA	Analysis of Variance
EDTA	Ethylene diaminetetraacetic acid
ATP	Adenosine triphosphate
BIND	Bilirubin-induced neurologic dysfunction

GIT	Gastrointestinal tract
AAP	American Academy of Pediatrics
IVIG	Intravenous Immunoglobulin
GLP	Good Laboratory Practice
IAMEBBC	Institutional Animal Medical Ethics Biosafety and Biosecurity Committee
Rh	Rhesus factor
AI	Adequate intake
UCB	Unconjugated bilirubin

LIST OF SIGNS AND SYMBOLS

Sign or Symbol	Meaning
Δ	Absorbance
-	En-dash used to denote bridged word
→	Arrow indicates direction or flow
=	Indicates equal to
–	Em-dash used to denote range, or used in place of other punctuation
×	Indicates multiplication of preceding and succeeding numbers
()	Denotes parenthesis or first-bracket
/	Indicates per
[]	Denotes bracket or third-bracket
±	Plus or minus
<	Less than
>	More than
&	And

ABSTRACT

Neonatal hyperbilirubinemia is a frequently encountered problem in 60%–80% of newborns worldwide. The burden is high in low-income and middle-income countries like Bangladesh. A recent report noted that at least 4,81,000 term/near-term neonates are affected by hyperbilirubinemia each year, with 1,14,000 dying and an additional 63,000 surviving with kernicterus. Under this circumstance, it is essential to tackle severe neonatal jaundice as one key component of optimizing neurodevelopmental outcome to achieve the sustainable development goals. Therefore, this study has designed to find the incidence and relation of neonatal jaundice with heavy metals such as copper, zinc and magnesium to inform child health policy regarding its prevention and management. About 594 neonates were collected from the outpatient and inpatient Department of Clinical Pathology(Laboratory medicine) and Neonatology at Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka. Infants with severe congenital malformation, sepsis, or birth asphyxia were excluded from this study. They were divided into case and control groups. This study was approved by the institutional ethical committees of Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh (Approval Memo no-82/320/IAMEBBC/IBSc, 20 August, 2017).

The mean age of the respondents was 3.08 and 3.21 days; the sex variable of the respondents were male 187 and 160 in case and control group respectively and female were 132 and 115 in case and control group respectively which was not significant. The mean weight of the respondents was 2.62±0.4 and 2.84±0.3 kilogram which was significant.

The concentration of serum bilirubin was significantly ($p<0.001$) higher (171.14 mg/L) in case group when compare to control group (35.42 mg/L). Because the liver function becomes progressively worse in case group.

The serum magnesium was 23.98 mg/L and 19.19 mg/L in case and control group, respectively. The concentration of serum magnesium is higher in case group when compared with control which is very significant ($p<0.001$). The level of serum

zinc was 0.50 mg/L in case group and 0.68 mg/L in control group. The serum zinc level is significantly ($p<0.001$) lower in case group when compared with control group. In case group the level of copper was 0.75 mg/L and in control group was 0.43 mg/L. It was significantly ($p<0.001$) higher in case group when compared with control group. There was a positive significant Pearson correlation ($r=0.817$, pvalue<0.001) between serum total bilirubin level and serum magnesium level in both groups. It means if serum bilirubin is increased, serum magnesium also increased in case group and control group. Relation between serum total bilirubin level and serum zinc level was negative significant Pearson correlation($r= -0.773$, p value <0.001) in both groups. It is justified that if serum bilirubin level is increased the serum zinc level also decreased in case group and control group. The positive significant Pearson correlation ($r=0.832$, p value <0.001) was in between serum total bilirubin and serum copper level in both groups and it can be said that if serum bilirubin level is increased, the serum copper level also increased in case group and control group.

So in this study, the heavy metal like zinc, copper and magnesium has relation with neonatal hyperbilirubinemia. If serum bilirubin is increased, serum magnesium and copper also increased but when serum bilirubin level is increased, the serum zinc level decreased in the patient of neonatal hyperbilirubinemia.

CHAPTER ONE

INTRODUCTION

1.1 Background

Neonatal hyperbilirubinemia is a condition when a newborn has an excessive amount of bilirubin in the blood that causes the yellowish discoloration of skin, eyes and mucous membrane of body. Neonatal jaundice is another a term of neonatal hyperbilirubinemia.[1]

Neonatal jaundice is a common clinical problem during the first week of neonatal period. Approximately 60% of full term and 80% of premature neonates develop clinical jaundice in the first week of life. Jaundice may become severe with a major risk of neonatal morbidity and mortality in a proportion of infants.[2]

The incidence of neonatal hyperbilirubinemia is more in East Asians and American Indians than Africans. In High Income Countries (HIC), the incidence of severe hyperbilirubinemia is about 31.6 per 100,000 live births, among them the range of 1.0–3.7 and 0.4–2.7 per 100,000 live births is acute and chronic bilirubin encephalopathy respectively. But this condition is entirely different in Low and Middle Income Countries (LMICs). Severe neonatal jaundice is 100 times more frequent than industrialized countries in Nigeria. Severity of neonatal jaundice may be related to differences in the distribution of the genetic variants and ethnic variability in bilirubin metabolism.[3]

In Bangladesh, bilirubin-induced mortality is significantly more important in early-neonatal than in neonatal.[4] The overall prevalence of neonatal hyperbilirubinemia in Bangladesh is about 33% and reported by various Indian workers varies from 4.6% to 77%.[3]

Between 3 and 6 postnatal days, plasma or serum bilirubin level observed in most infants is optimum and the risk of severe neonatal jaundice (NNJ) is highest in this time. For prevention of bilirubin-induced mortality the timely detection, monitoring, and treatment within that 3-6 post natal period is very important. In the birth centre of many developed countries infants are screened after their birth and keep them under observation for any severe hyperbilirubinemia even after discharge.

However, this screening facilities and resource is very limited in low and middle income countries because a good number of births occur outside of hospital, mothers are thus heavily burdened with the responsibility of strictly recognizing neonatal hyperbilirubinemia among their newborns.

There are three levels of barriers to effective intervention in upward countries with significant stagnant and potentially unsafe exchange transfers, as well as bilirubin-induced mortality. First, home-based therapies before being presented to the hospital. Second, the lack of adequate resources, financial assistance and health facilities. Third, the lack of benefits from poorly maintained phototherapy devices for fast, routine bilirubin purposes.

Generally, there is no intervention require for neonatal jaundice in benign condition. Most cases of neonatal jaundice found physiological jaundice and does not require intervention and do not have serious consequences.[5] Five to ten percent neonatal jaundice need treatment because high bilirubin levels can be toxic for central nervous system and may cause behavioral and neurological impairment even in term newborns.

Birth weights, gestational age, premature rupture of membranes, maternal infectious diseases or other illness during pregnancy, having different sources of origin of food are responsible for Neonatal jaundice.[6]

Zinc is the vital body elements which have role in natural growth, development, and many natural performances of human body. Zinc deficiency is a common health problem in pregnant women and that is high risk for neonates as well as mother. During pregnancy, zinc deficiency may cause growth impairment, spontaneous abortion, congenital malformations, intrauterine growth retardation (IUGR), low birth weight (LBW), preeclampsia, premature labor, prolonged labor, postpartum bleeding, delayed neurobehavioral development, delayed immune system development, and leads to increase of mortality rate.[7] Deficiency of serum zinc can lead to a deficiency some enzymes that have an important role in bilirubin metabolism. Structural defects in the erythrocyte membrane can also be caused by hemolysis.[2]

Copper is essential for the prevention of anemia and leucopenia due to active component of several enzyme systems, including cytochrome oxidase and superoxide dismutase. Occasionally, the high level of serum copper may be accountable for neonatal jaundice because of slight breakdown of erythrocyte.[8]
Magnesium is an essential co factor for cellular respiration, glycolysis, and trans-membrane transportation of sodium and calcium. It is also affect the enzyme activity by binding of ATP requiring enzyme and the active site of enzyme (pyruvate kinase, enolase), by causing conformational changes during the catalytic process (Na-K-ATPase), and by promoting aggregation of multi enzyme complexes.[9] In elderly, hypomagnesaemia is rare and mostly seen in renal failure.
This may be a positive correlation between ionized magnesium level and severity of hyperbilirubinemia patients in neonate.[10]

Neonatal jaundice is a common cause of morbidity in neonates of Bangladesh. However; more information or data is not obtainable to find out the exact causes or situation of newborn jaundice.[11] So this present study was carried out to find out the characteristics of the neonatal jaundice and any relation of heavy metals like zinc, copper and magnesium with newborn hyperbilirubinemia, in Bangladesh.

1.2 Statement of the Problem

The first 28 days of life is the most vulnerable time for a child's survival. Children face the highest risk of dying in their first month of life at an average global rate of 18 deaths per 1,000 live births in 2017. In the first month of life in 2017, 2.5 million children died globally. Approximately 7,000 neonatal deaths every day and most of which occurred in the first week. Out of them, 1 million dying on the first day and close to 1 million dying within the next six days.[12] During this period, the neonates are at risk of acquiring many problems. Among these, the major health problems are jaundice, infections, nutritional deficiency, trauma and regulation of body temperature. Epidemiological evidence suggests that severe neonatal jaundice results in substantial morbidity and mortality. It is a significant cause of cerebral palsy, long-term neurocognitive sequelae, non-syndromic auditory neuropathy, deafness and learning difficulties.[13] The problem is high in low-income and middle-income countries like Bangladesh. Each year 4,81,000 term or near-term neonates are affected by hyperbilirubinaemia, out of them $1{,}14{,}000$ are dying and additional $63{,}000$ are surviving with kernicterus a recent study noted this sceniario.[14] Under this circumstance, it is essential to tackle severe neonatal jaundice as one key component of neurodevelopmental outcome to achive the sustainable development goals (SDGs). Therefore, this study has designed to find the incidence and relation of neonatal jaundice with heavy metals such as copper, zinc and magnesium to looks child health safety, diseases prevention and management.

1.3 Rationale

This study is an attempt to assess the relation of heavy metals such as copper, zinc and magnesium with neonatal jaundice in Bangladesh. The findings may be suggestive for application of the prevention and treatment of neonatal jaundice. This study would be beneficial for individual sufferers, and vis-à-vis the nation and overall the social development of the country.

1.4 Hypothesis

The study was conducted keeping in mind the hypothesis −

The selected heavy metals may have a relation with neonaetal jaundice in term of statistical significance (using *t* test and chi-square test) when compared between two groups. The specific parameters are serum bilirubin, serum copper, serum zinc and serum magnesium level compared with the effects of case group and control group.

1.5 Objectives

General Objective :

To determine the level of serum zinc, serum copper, serum magnesium in both neonatal hyperbilirubinemia patients and healthy neonates.

Specific objectives:

The study will be undertaken with the following specific objectives-

- To assess the level of zinc in neonatal hyperbilirubinemia.
- To assess the level of magnesium in neonatal hyperbilirubinemia.
- To assess the level of copper in neonatal hyperbilirubinemia.
- To assess the level of zinc, magnesium and copper levels in healthy neonates.
- To find out the correlation between the level of zinc, magnesium and copper with hyperbilirubinemia.

1.6 Operational definitions

Neonate: A neonate is a child under 28 days of age.

Hyperbilirubinemia: Hyperbilirubinemia refers to an excessive level of bilirubin accumulation in the blood and is characterised by yellowish discolouration of the skin, sclera, mucous membrane and nails.

Trace elements: A chemical element required in minute quantities of physical functitioning, especially a micronutrient (such as iodine, iron, and zinc) with an optimum daily intake of typically less than 100 mg a day.

Low birth weight (LBW): Birth weight of less than 2500 gm.

CHAPTER TWO

LITERATURE REVIEW

The word Jaundice is actually a derivative of French word '*Jaune'* which means 'yellow'. Jaundice is defined as a condition where the skin, eyes, mucous membranes and body fluids turn yellow due to a high level of bilirubin. Normally, bilirubin is delivered from the bloodstream into liver. Then, it passes through tubes called bile ducts. These ducts carry bile into small intestine. Eventually, bilirubin is passed out from body through urine or stool. The bilirubin may be in conjugated or unconjugated form.[15]

Jaundice is the result of hyperbilirubinemia, it occurs when there is an imbalance between bilirubin production, conjugation and elimination. The breakdown of red blood cells (RBC) and haemoglobin cause unconjugated bilirubin to accumulate in the blood. Unconjugated bilirubin formed conjugated bilirubin after binding to albumin which then transported to the liver. It is eliminated via urine and faeces.[16]
Jaundice is classified as unconjugated, hepatocellular, or cholestatic. The first type is unconjugated, or hemolytic, jaundice, appears when the amount of bilirubin produced from hemoglobin by the destruction of red blood cells or muscle tissue exceeds the normal capacity of the liver to transport it or when the ability of the liver to conjugate normal amounts of bilirubin into bilirubin diglucoronide is significantly reduced by inadequate intracellular transport or enzyme systems. The second type, hepatocellular jaundice, arises when liver cells are damaged so severely that their ability to transport bilirubin diglucoronide into the biliary system is reduced, allowing some of the yellow pigment to regurgitate into the bloodstream. The third type, cholestatic, or obstructive, jaundice, occurs when essentially normal liver cells are unable to transport bilirubin either through the hepatic bile capillary membrane, because of damage in that area, or through the biliary tract, because of anatomical obstructions such as gallstones or cancer.[17] The differential diagnosis for jaundice is age-specific this review addresses the causative conditions in infants beyond the newborn period, older children, and adolescents.

2.1. Bilirubin

Bilirubin is an endogenous compound. It can be toxic in neonates. The recent study recognized that unconjugated bilirubin (UCB) exerts a strong antioxidant activity. Bilirubin is the ultimate breakdown product of haemoglobin and important diagnostic marker of liver and blood disorders. It metabolism is complex, which is important in relation to several processes of drug metabolism.[18]

2.1.1 Formation of bilirubin

The majority of bilirubin (80%) is produced from the degradation of hemoglobin from erythrocytes undergoing normal (removal of aged or effete cells) or abnormal destruction (i.e. intravascular or extravascular hemolysis) within mononuclear phagocytes (principally splenic, hepatic and bone marrow macrophages). A small percentage (20%) is derived from the catabolism of various hepatic hemoproteins (myoglobin, cytochrome P450) as well as from the overproduction of heme from ineffective erythropoiesis in the bone marrow. Within macrophages, a free heme group (iron plus porphyrin ring) is oxidized by microsomal heme oxygenase into biliverdin and the iron is released (the iron is then stored as ferritin or released into plasma, where it is bound to the transport protein, transferrin). Biliverdin reductase then reduces the green water soluble biliverdin into unconjugated (water insoluble but lipid soluble) bilirubin. Heme oxygenase is also located in renal and hepatic parenchyma, enabling these tissues to take up heme and convert it to bilirubin.[19]

2.1.2 Metabolism of bilirubin

Bilirubin is a product of heme catabolism. Red cell hemoglobin accounts for approximately 85% of all bilirubin. In newborns, the normal hemoglobin level is 15-18 mg/dl. The rate of neonatal RBC destruction is higher than in adults resulting in greater quantity of hemoglobin release. Excessive bruising from birth trauma or abnormal blood collections such as in a cephalohematoma may further add to the rate of RBC destruction and bilirubin formation. Heme is catabolized to unconjugated bilirubin in the reticuloendothelial system. Unconjugated bilirubin is bound to albumin in the plasma and transported bound to albumin to the liver and is conjugated with glucuronic acid in the hepatocytes; the conjugation is catalyzed by

glucuronyl transferase. Conjugated bilirubin is secreted into the bile and enters the duodenum. In the small bowel, some of the bilirubin is hydrolyzed to yield unconjugated bilirubin and glucuronic acid. Most unconjugated bilirubin is excreted in the stool, but some is reabsorbed and returned to the liver for re-conjugation (enterohepatic circulation). The level of glucuronyl transferase is initially low in the newborn and any increase in the rate of bilirubin formation can overwhelm the capacity to conjugate, thus resulting in elevated bilirubin levels.[20]

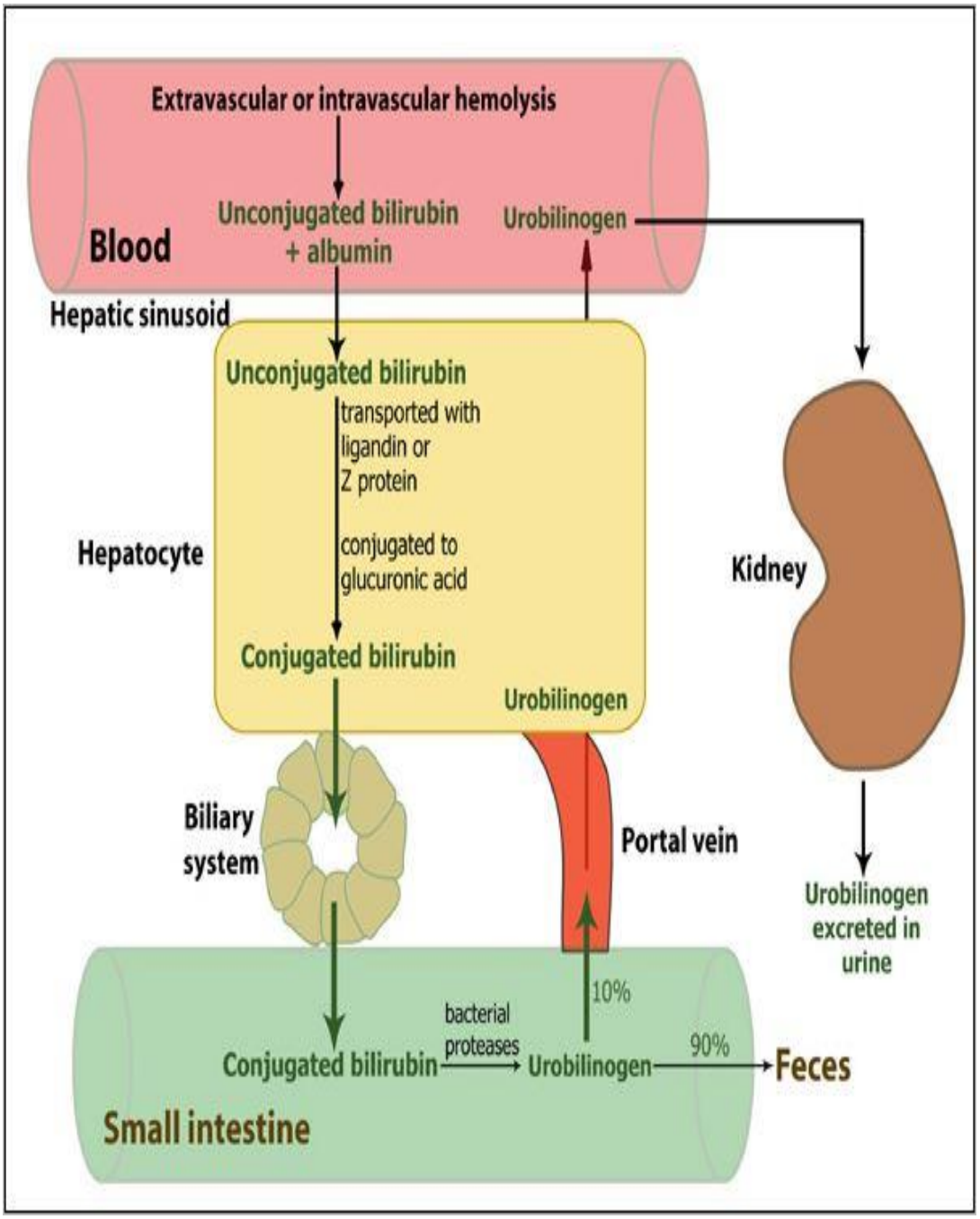

Fig.2.1 Bilirubin Formation and Metabolism

2.2. Neonatal Jaundice

Jaundice is a common clinical sign in newborns, especially during the first 2 weeks after birth. The first description of neonatal jaundice and bilirubin staining of the newborn brain goes back to the eighteenth century.

2.2.1. History of newborn jaundice

Severe form of neonatal was first identified in 19th century. During the past 100 years, the following major themes have dominated thinking about the pathogenesis and treatment of newborn jaundice.

- Understanding the chemical basis of jaundice or identification of' the substance causing jaundice as bilirubin.
- Conceptualization of the biochemical process of metabolism of bilirubin and transport, in liver and blood.
- Development of Rh disease
- Of kernicterus but still least understood.
- unconjugated hyperbilirubinemia

Virchow in 1847, isolated bilirubin and in 1950 it was confirmed that heme is the source of bilirubin which conjugates with glucuronide. Lathe and Walker in 1957 showed that the newborn deficient in glucuronyl transferase (hepatic enzyme responsible for the formation of the glucuronide conjugate of bilirubin) was responsible for physiologic jaundice of the newborn. This point is still under investigation.[21]

The finding of jaundice on physical examination is an indicator of hyperbilirubinemia. Hyperbilirubinemia is defined as a total serum bilirubin level greater than 1.5 mg/dL. In newborns, serum bilirubin universally exceeds this level for physiological reasons during the transitional period after birth. Jaundice becomes evident when the total serum bilirubin level reaches 5 mg/dL. More than 60% of healthy newborns develop neonatal jaundice and receive diagnoses of neonatal hyperbilirubinemia during the first week after birth. In a more recent study, neonatal jaundice affected 84% of neonates born at least 35 weeks of gestation. Jaundice usually begins on the face and

progresses in a cephalocaudate fashion, for unknown reasons. The total bilirubin level roughly correlates with progression of jaundice (face, 4–8 mg/dL; upper trunk, 5–12 mg/dL; lower trunk, 8–16 mg/dL; soles of the feet,>15 mg/dL).[22]

2.2.2. Prevalence of Neonatal Jaundice

Globally, 2.5 million children died in the first month of life in 2017 alone approximately 7,000 neonatal deaths every day most of which occurred in the first week, with about 1 million dying on the first day and close to 1 million dying within the next six days. Neonatal mortality declined globally and in all regions but more slowly than mortality among children aged 1–11 months or 1-4 years in most cases. Globally, the neonatal mortality rate fell by 51 per cent from 37 deaths per 1,000 live births in 1990 to 18 in 2017, a smaller reduction in mortality than among children aged 1-59 months (63%).[23] The mortality rate in the late neonatal period was in the late-neonatal period was about 187.1 per 100000 and ranked ninth globally. It is reported that from the study of hyperbilirubinemia 60% of newborns suffer from clinical jaundice in United States.

The mortality rankings among the 10 countries that frequently account for the largest number of neonatal deaths worldwide are in Nigeria, the Democratic Republic of the Congo, Ethiopia, Angola, Kenya (Sub-Saharan Africa); India, Pakistan, and Bangladesh (South Asia); China and Indonesia (East or Southeast Asia).In view, bilirubin induced mortality was consistently among the top 15 causes of neonatal mortality in these 10 countries. Also, neonatal jaundice mortality was uniquely more prominent in the late neonatal than early neonatal period in Bangladesh.[4]

2.2.3. Reducing the Burden of Neonetal Jaundice in High Burden Countries

Like most neonatal diseases, neonatal jaundice cannot be prevented.Timely effective phototherapy can reduce bilirubin-induced mortality in developed countries. Mothers should be trained to detect the onset of severe neonatal jaundice and seek professional care immediately also should be aware of delayed or inappropriate treatment for neonatal jaundice). To reduce the chance of hemolytic jaundice it is essential to test for glucose 6-phosphodehydrogenase deficiency, About 10.8 million

children under the age of five die worldwide in each year, and 38% of these deaths are reported to occur in the first month of life.

Similarly, recent global estimates put the number of neonatal deaths at approximately 3.6 million. The global burden of neonatal hyperbilirubinemia affects most of the world's low-income countries(South Asia and sub-Saharan Africa).
High level of hyperbilirubinemia in Latin America, sub-Saharan Africa, and South Asia accounted for 4%, 32%, and 39%, respectively, for a combined outbreak of 10/100,000 live births.

The differences in the prevalence of neonatal jaundice may be related to the difference in bilirubin metabolism in the early neonatal period. A study conducted in 2013 to determine the global estimates of extreme hyper bilirubinemia found that sub-Saharan Africa accounts for 35% of the kernicterus cases. In Namibia little is known on the risk of neonatal jaundice and the extent of its contribution to neonatal disease and death.[24]
Today, severe hyperbilirubinemia is the most common cause of neonatal insufficiency in North America. It is important to identify clinically important newborns at risk of hyperbilirubinemia before they are discharged from the hospital.[25]

2.2.4. Causes of neonatal hyperbilirubinemia[26]

a) Conjugated hyperbilirubinemia:

- **Intrahepatic causes:**
  - Parenchymal disease**:**
    - Infections: Toxoplasmosis, rubella, cytomegalovirus, herpes, hepatitis B,septicemia due to bacterial infections.
    - Metabolic disorders: Alpha-1-antitrypsin deficiency, cystic fibrosis, tyrosinemia, hereditary fructose intolerance, galactosemia.
    - Cryptogenetic neonatal hepatitis syndrome.
    - Benign recurrent cholestasis syndrome.

- Familial neonatal chollestasis with fatty infiltration of liver and bleeding diathesis.

- Intrahepatic biliary hypoplasia or atresia; In these patients cholesterol level is above 250mg/dl and serum alkaline phosphatase is raised more than 3 fold.
  - This condition may be associated with extrahepatic biliary atresia.
  - In such cases biliary cirrhosis is delyed even if the biliary obstruction is unrelieved. Liver biopsy may show support of selerosing cholangitis.
  - Arterriohepatic dysplasia; These patients may show prominent forehead deep-set eyes and a straight nose in the same plane as the forehead, vertebral arch anomalies, hypogonadism and congenital heart disease such as pulmonary stenosis.
  - Cystic dilatation of intrahepatic bile duct. jaundice may appear at any age but it generally presents in order children, diagnosis is alleged on ultrasonography.

- **Extra hepatic causes:**
  - Extrahepatic biliary atresia: There is complete obstruction of bile duct in branches of the right or left extrahepatic ducts or regular bile duct. The scope of obstruction may be variable and may include the entire biliary tree. Intrahepatic bile ducts are proliferated and there is periductular fibrosis.
  - Choledochal cyst.
  - Biliary atresia associated with polysplenea.
  - Inspissated bile syndrome following extreme hemolysis.

b) Unconjugated hyperbilirubinemia :

- Physiologic: Hepatic immaturity
- Excessive red cell devastation
  - Rh inappropriateness
  - ABO incompatibility

- Glucose-6-phosphate dehydrogenase deficiency
- Pyruvate- Kinase deficiency
- Disseminated intravascular coagulation
- Sphercytosis
- Cephalhematoma
- Drugs: vitamin k, sulphametozazole

- Metabolic factors
  - Familial transitory hyperbilirubinemia
  - Criggler and Najar syndrome: Type-1. deficiency of glucoronyl transferase; autosomal recessive; starts early in the life, often increase kernicterus. Type-II, autosomal dominant, with variable penetrance. Onset may be delayed, severity is variable.
  - breast milk inhibitor.3 alpha, 20 beta pregnanedial inhibits glucoronyl transferase
  - Anoxia
  - Respiratory pain
  - Starvation
  - Maternal diabetes
  - Pyloric stenosis
  - Drugs
- Mixed hemolytic and hepatotoxic disease; septicemia, intrauterine infections such as cytomegalovirus toxoplasmosis, rubella, drugs.
- Hepatocellular: neonatal hepatitis, biliary hypoplasia, biliary atresia, Galactosemia
- Enterohepatic recirculation: The physiological jaundice may be incompletely be qualified to enterohepatic recirculation of bilirubin. The bilirubin may be conjugated by the liver and then excreted in the gut. As there is no bacterial action in the intestine in the first few days of life the bilirubin excreted in the gut can not be converted into urobilinogen. Physiologic hyperbilirubinemia may be exaggerated in the following circumstaces;

- Prematurity, rate of maturation of liver funtoins is slower in the premature infant. The highest level of bilirubin is reached on the 7^{th} or 8^{th} day and the jaundice disappears by 10^{th} day indirect bilirubin level may be elevated to 15mg/dl.
- Hypoxia and circulatory insufficiency: in circulatory insufficiency, bigger number of erythrocyte are sequestrated in the lungs. The cells hemolyse and increase the bilirubin load. Besides this hepatic perfusion is also compact due to circulatory insufficiency.
- Drugs: Novobiocin aggravates the physiological jaundice by inhibiting the metabolism of bilirubin. Salicylates and long acting sulphonamides dissociate bilirubin from the albumin bilirubin complexby aggressive reserve. Vitamin k a physically powerful oxidant increases hemolysis. It also blocks Y proteins necessary for bilirubin uptake by the cells. The follwing drugs given to the mother decrease serum bilirubin in the baby. Narcotics, barbiturates, aspirin, phenytoin and reserpine.
- Role of cephalhematoma: Each gm of hemoglobin liberates 34gm bilirubin on hemolysis. Cephalhematoma is an important cause of the amplified bilirubin load.
- Role of infections: Intrauterine infections with toxoplasma gondi
- Cytomegalovirus or syphilis cause hepatocellular damage leading to the faulty secretion of the bilirubin. Bacterial infections are the major cause of icterusafter 3 days of life.

2.2.5 Risk factors [27]

- Major risk factors
  - Predischarge total serum bilirubin or transcutaneous bilirubin level in the high-risk zone
  - Jaundice observed in the 1st 24 hours.
  - Blood group incompatibility with positive direct antiglobulin test
  - Other known hemolytic disease (glucose-6-phosphate dehydrogenase deficiency),
  - Elevated end-title CO concentration
  - Gestational age 35-36 week
  - Previous sibling received phototherapy
  - Cephalohematoma or significant bruising
  - Exclusive breastfeeding, particularly if nursing is not going well and
  - Weight loss is excessive
  - East Asian race
- Minor risk factors
  - Predischarge total serum bilirubin or transcutaneous bilirubin level in the high intermediate-risk zone
  - Gestational age 37-38 week
  - Jaundice observed before discharge.

2.2.6 Physiology of neonatal unconjugated hyperbilirubinemia

Bilirubin is the end product of heme catabolism. The heme comes mainly from aging red blood cells (RBCs), but muscle myoglobin and some liver enzymes such as cytochromes and catalases are a partial source. Fetal and adult hemoglobin are structurally different. Together with a higher hemoglobin concentration, this explains the increased oxygen-carrying capacity in the fetus. Postnatally, these mechanisms are no longer needed. Production of adult hemoglobin starts in the last trimester of pregnancy, and in the absence of certain hemoglobinopathies the production of fetal hemoglobin is turned off at birth. By 20 weeks of gestation IXα constitutes 6% of bilirubin in bile, IXβ 87%, IXγ 0.5%, and IXδ 6%. By 38 weeks gestation, bilirubin-IXα has replaced IXβ as the main isomer. In fetal hemolytic disease, such as Rhesus

immunization, the concentration of total serum bilirubin (TSB) can increase from 1.5 mg/dL at 20 weeks of gestation to 4.1 mg/dL at 32 weeks. Heme oxygenase found in the reticuloendothelial system, tissue macrophages and in gut mucosa catalyzes breakdown of heme.

2.2.7 Pathophysiology of neonatal jaundice[28]

Bilirubin is formed in the liver and spleen then it passes through several processes in order to be metabolized. Jaundice develops due to increase the level of bilirubin and deposition under the skin and cause the yellow discoloration of the skin. Pathogenesis of neonatal jaundice includes physiologic process of bilirubin accumulation or pathological mechanism. The pathological jaundice may be acquired or inherited. Acquired neonatal jaundice includes Rh hemolytic disease, ABO incompatibility disease, and hemolytic disease due to G6PD enzyme deficiency. Inherited neonatal jaundice is due to defect of one of the processes of bilirubin metabolism and it concludes some inherited syndromes.

2.2.8. Clinical presentation

History:

The family history of jaundice, anemia, splenectomy or metabolic disorders is significant in most cases. A history of sexual intercourse with jaundice may suggest blood group incompatibility, breast milk jaundice, G6PD deficiency.

Neonatal jaundice increases with a history of obstetric diabetes and infection. In infant hyperbilirubinemia may be due to delivery trauma, asphyxia, and delayed cord clamping and premature infancy.

Poor breastfeeding can result in "starvation jaundice".Decreased gastric motility and obstruction.Clinical symptoms such as nausea and lethargy should be examined. Metabolic disorders, infections and intestinal obstruction may be present in these situations.

2.2.9. Diagnosis[29]

The American Academy of Pediatrics (AAP) recommends that all babies be checked for jaundice after birth while in the hospital.

Physical examination: All jaundiced babies require an assessment including history and a full clinical examination.

Jaundice: Jaundice appears cephalocaudal depending on severity and regresses in the reverse order.

Intake/output: Feeding assessment, assess weight in first week of life

Urine: Dark urine may be indicative of conjugated hyperbilirubinaemia

Laboratory Examinations:

1. Serum total and conjugated bilirubin level
2. Liver enzymes:
    - SGPT/ALT (Serum transaminase) alanine transaminase
    - SGOT/AST (Serum transaminase) aspartate transaminase
    - Prothrombin time and partial thromboplastin time
3. In case of phototherapy:
    - ABO /Rhesus blood group of baby and mother.
    - Complete blood count, peripheral blood film.
    - Reticulocyte count.
    - Direct coombs test.
    - Serum albumin
    - Serum electrolyte
    - Serum creatinine
    - Cross match (baby and mother's blood) and screening test.
    - Glucose-6-phosphate dehydrogenase screen.
    - Redcell enzyme assays.
4. Others investigations
    - CRP (C-reactive protein)
    - Thyroid function test.
    - Urine: Urobilinogen, bile salts

- Stool: Stercobilinogen
- Blood ammonia levels
- Serum lactate and pyruvate
- Serum and urine amino acids
- Serum glucose levels
- TORCH Pannel
- PCR (Polymerase chain reaction) based diagnostic studies are extremely helpful and specific
- $Alpha_1$-antitrypsin serum level

5. Ultrasonography and Percutaneous liver biopsy.

2.2.10. Management

Key principles of jaundice management include prevention, identification, and evaluation of at-risk children with hyperbilirubinemia and phototherapy

Treatment of jaundice is grouped into two main categories: phototherapy and pharmacological therapy. Phototherapy is further classified into conventional, intensive, and exchange transfusion, whereas pharmacological therapy is further grouped into IV immunoglobulins, phenobarbitone, and metalloporphyrins.[30]

Phototherapy:

Phototherapy provides an easy and safe method to treat hyperbilirubinemia with minimal side effects. Its efficacy mainly depends on the exposure to the phototherapy; for example, single surface phototherapy is significantly less effective than double surface phototherapy. Moreover, the use of phototherapy continuously is associated with better outcomes than its use intermittently. It is recommended not to interrupt phototherapy except during breastfeeding.

Pharmacological treatment: Pharmacological treatment of neonatal jaundice is as follows.

Phenobarbitone: This agent work by decreasing the processing of bilirubin that includes the uptake, conjugation, and excretion of the bilirubin by the liver. This will eventually lead to a significant reduction in the levels of bilirubin in the blood.

However, phenobarbitone has a slow onset, and takes a few days to effectively work. It has been found to lead to a significant improvement of jaundice in neonates with hemolytic jaundice following three to five days of treatment. It also has a relatively safe profile, with minimal adverse events. The use of phenobarbitone in the management and treatment of neonatal jaundice is supported by solid evidence and large studies.

Intravenous Immunoglobulin (IVIG): High dose IVIG (0.5-1 gr/kg) has shown to be effective in decreasing the needs of exchange transfusion and phototherapy in babies with Rh hemolytic disease.

Metalloporphyrins: These drugs are still in the experimental phases with no strong evidence supporting their use. However, results are promising for the management and treatment of both hemolytic and nonhemolytic jaundice in the neonates. Moreover, they have been shown to be associated with relatively safe profiles and minimal adverse events.

2.2.11. Complications of unconjugated hyperbilirubinaemia

Acute and chronic bilirubin encephalopathy: In hyperbilirubinaemia unconjugated bilirubin is deposited in and stains the auditory pathways, basal ganglia and oculomotor nucleus, resulting in acute and then chronic bilirubin encephalopathy or kernicterus.

Bilirubin induced neurologic dysfunction: Bilirubin induced neurologic dysfunction is a syndrome of subtle bilirubin neurotoxic disorders that can occur in the absence of kernicterus.

2.3. Trace elements

More than 50 chemical elements are found in the human body which is required for growth, repair and regulation of vital function they are a) major minerals such as calcium, phosphorous, sodium, potassium and magnesium b) trace elements such as iron, iodine, fluorine, zinc, copper, cobalt, chromium, manganese, molydecem, selenium nickel, silicon & vanidicem.[31] Trace elements play a important role in maintenance of a healthy state of an organism. Abnormalities in trace element homeostasis may result in the development of pathologic states and diseases.[32]

Essential trace elements of the human body include zinc (Zn), copper (Cu), selenium (Se), chromium (Cr), cobalt (Co), iodine (I), manganese (Mn), and molybdenum (Mo). Although these elements account for only 0.02% of the total body weight, they play significant roles, e.g., as active centers of enzymes and as trace bioactive substances. Like vitamins, trace elements originally viewed as nutrients. Trace elements exert pharmacological actions if they are ingested in amounts of ten times higher than the nutritional requirements. Excessive ingestion of trace elements as medicines has also been reported, which may lead to poisoning. Congenital abnormalities of trace element metabolism are rare. Abnormal intestinal absorption or disturbed transport of absorbed trace elements more often lead to deficiency of trace elements.

Acrodermatitis enteropathica due to disturbed zinc absorption and Menkes disease due to abnormal copper transport through the intestinal mucosa are some examples of such conditions. If the uptake site of trace elements into an active form is disturbed, the trace element is pooled there, causing excess of the element. In cases of Wilson disease characterized by disturbed uptake of copper into ceruloplasmin, tissue damage and fibrosis due to copper occur in the liver and other sites.[33]

2.3.1. Zinc

Zinc is an essential nutrient for all forms of our life. Its importance lies in the fact that many body functions are linked to Zinc containing enzymes. It as a trace element has indispensable role in human health and diseases.[34] Zinc plays a critical role in normal functioning and is integrated with several enzyme systems of our body.

Gene expression, cell division, immunity, and reproduction are important biological functions of zinc elements.[35]

Dietary sources of zinc and recommended dietary allowance (RDA):

Zinc is an essential trace element of our body. RDA of zinc is about 10-15 mg/day for adult and 15-20 mg/day for pregnant women. Major sources of zinc include meat, liver, milk and dairy products. Vegetables sources are legumes, pulses, nuts, beans & spinach etc. Animal sources are better absorbed than vegetable sources.[36]

Table 2.1: Recommended Dietary Allowances (RDAs) for Zinc

Age	Male	Female	Pregnancy	Lactation
0–6 months	2 mg*	2 mg*		
7–12 months	3 mg	3 mg		
1–3 years	3 mg	3 mg		
4–8 years	5 mg	5 mg		
9–13 years	8 mg	8 mg		
14–18 years	11 mg	9 mg	12 mg	13 mg
19+ years	11 mg	8 mg	11 mg	12 mg

* Adequate Intake (AI)

Distribution of zinc: Zinc is present in all tissue and fluids of the body. The total body content is about 2.4 grams (30-40 mmol) in adult, of which skeletal muscle accounts for approximately 60% and bone for about 30%. Plasma zinc represents only about 0.1% of total body content; it has a rapid turnover and level appear to be under close homeostatic control. The majority of zinc in plasma is bound to albumin which acts as the transport vehicle.[37]

Functions:

(a) Catalytic function: Nearly 100 specific enzymes depend on zinc for catalytic activity. Zinc metallo-enzymes include the ribonucleic acid (RNA), polymerases, alcohol dehydrogenase, carbonic anhydrase, alkaline phosphatase.

(b) Regulator of gene expression.

(c) It influences both apoptosis and protein kinase C activity.

(d) Zinc provides structural role for binding of tyrosine kinase to T-cell receptors, CD4 and CD8, which are required for T-lymphocyte development and activation.

Physiology of Absorption, Metabolism, and Excretion:

Digestion produces the opportunity for Zinc to bind to exogenous and endogenous elements in the intestinal lumen, including peptides, amino acids, nucleic acids and other organic acids and inorganic anions such as phosphate. The majority of Zinc is absorbed by the small intestine through a transcellular process with the jejunum being the site with the greatest transport rate. Zinc secretion into and excretion from the intestine provides the major route of endogenous Zinc excretion. In contrast to excretion of Zinc via other routes, excretion of endogenous zinc via the intestine is a major variable in the maintenance of zinc homeostasis and is strongly correlated with absorbed zinc.[38]

Deficiency of zinc:

Human zinc deficiency is mainly caused by low Zinc in the diet; high fiber content decreases the availability of Zinc for intestinal absorption. The main clinical features of Zinc deficiency include growth retardation, delay in skeletal maturation, testicular atrophy and hepato-splenomegaly. Other manifestations of human Zinc deficiency include susceptibility to infection, impaired wound healing, scaly dermatitis and diarrhoea.[39]

2.3.2. Copper

Copper is an important trace element that is vital to the health of all living things (humans, plants, animals, and microorganisms). In humans, Copper is very essential to the proper functioning of organs and metabolic processes. The human body has complex homeostatic mechanisms which attempt to ensure a constant supply of available Copper, while eliminating excess copper whenever this occurs. However, like all essential nutrients, too much or too little nutritional ingestion of copper can result in a corresponding condition of copper excess or deficiency in our body, each of which has set of adverse health effects.[40]

Homeostasis[41]

Deficiency condition may develop at any time. If more copper is ingested, an excess condition can result. Both of these conditions, deficiency and excess, can lead to tissue injury and other diseases. However, in the human body copper is absorbed, transport, distribute, and stored by a complex homeostatic processes and at the same time is able to excrete in our bodies to avoid excess levels of copper. If inadequate amounts of copper are consumed for a short period of time, the copper stores in the liver will decrease. If this decline continues, then copper is an unbalanced copper in a wide range of health needs for the body of a healthy person.

Cupro-enzymes involved in redox reaction that is common in metabolic processes (mitochondrial respiration, melanin synthesis, and cross-linking of collagen etc.). Copper-zinc superoxide dismutase (CU, ZN-SOD) enzyme plays a role in iron homeostasis. Copper is an essential trace mineral that cannot be formed by the human body and must be ingested from dietary sources.

Dietary sources of copper and recommended dietary allowance [42]

Beside dietary sources tap water and other beverages can also be sources of copper. Daily requirement of children (1-8 yrs.) ranging from 300-440 mcg and for adult about 900mcg

Copper and health benefits [40]

Copper is incorporated into a variety of proteins and metallo-enzymes which perform important metabolic functions; the micronutrient is necessary for the proper growth, development, and maintenance of bone, connective tissue, brain, heart, and many other body organs also. Copper is act in the formation of red blood cells, the absorption and utilization of iron, the metabolism of cholesterol and glucose, and the synthesis and release of life-sustaining proteins and enzymes. These enzymes in turn produce cellular energy and regulate nerve transmission, blood clotting, and oxygen transportation., and the normal act of the heart and immune systems in infants. Infants have special biochemical mechanisms for Copper stimulates the immune system to fight infections, to repair injured tissues, and to promote healing purpose.

Copper also helps to neutralize “free-radicals”, which can cause severe damage to cell properties. Copper is essential for the normal growth and development of human fetuses, infants, and children also. The human fetus accumulates copper rapidly in its liver during the third trimester of pregnancy period. At birth, a healthy infant has 4 times the concentration of cu than a full-grown adult. Human milk is comparatively low in copper, and the neonate’s liver stores falls rapidly after birth, supplying copper to the fast-growing body during the breast-feeding. These supplies are necessary to carry out such metabolic functions eg. cellular respiration, melanin pigment and connective tissue synthesis, iron metabolism, free radical defense, gene expressionmanaging copper in their bodies while permanent lifelong mechanisms develop and mature.

2.3.3. Magnesium

Magnesium is the 4th most abundant cation in the body and its vast majority is stored intracellularly. It is however, the extracellular concentrations of the mineral is of interest to the clinician due to its association with symptoms and signs. The major organs involved in magnesium homeostasis are the gut, bone, and kidney, but the regulators affecting these organs at the cellular level are not yet fully understood by well documentation. Hypermagnesemia is rare and is seen mostly in those with renal failure and in the elderly person.[43]

Distribution of magnesium:

The total body magnesium concentration is approximately 2000 mEq, or 25 g. Only a small fraction (approximately 1%) of the body magnesium is present in the extracellular fluid compartment and approximately 60-65% of the total body Magnesium is found in bone. Most of the magnesium in bone is associated with apatite crystals. A significant amount of the Magnesium in bone is present as a surface-limiting ion on bone crystals and is freely exchangeable. Approximately 20% of the total body magnesium is localized in the muscle. The remaining 20% is found in other tissues of the body.[44]

Function of magnesium: Magnesium is an essential mineral with multiple functions in the human body. This includes-

(a) Structural role in bones, cell membranes, chromosomes. It is needed for more than three hundred metabolic reactions including magnesium dependent chemical reactions required to metabolize carbohydrates and fats in the production of adenosine triphosphate (ATP), the "energy currency" of the body.

(b) It is required for the synthesis of nucleic acids, proteins, carbohydrates, lipids and the antioxidant glutathione.

(c) It is also required for transporting potassium and calcium ions across cell membranes.

(d) Conduction of nerve impulses, muscle contraction, normal heart rhythm, cell signaling and cell migration.[45]

Absorption of magnesium:

A small amount of magnesium, on the order of 15-30 mg/d, is secreted in the gastrointestinal tract. Magnesium homeostasis involves the kidney, small bowel and bone. However, most evidence suggests that Magnesium is absorbed mainly by ionic diffusion and "solvent drag" resulting from the bulk flow of water. At low intraluminal concentrations, magnesium is absorbed primarily through the active cellular route and with increasing concentrations, through the paracellular pathway (Kevin, Esther and Eduardo, 2009).[30]

Dietary sources of magnesium and recommended dietary allowance[46]

Magnesium is widely distributed in plant and animal foods and in beverages. Foods containing dietary fiber provide magnesium. Magnesium is added to some breakfast cereals and other fortified foods also. Some types of food processing eg. refining grains in ways that can remove the nutrient-rich germ and bran, lower magnesium content substantially. Tap, mineral, and bottled waters can also be sources of magnesium, but the amount of magnesium in water varies by source and different brands (ranging from 1 mg/L to more than 120 mg/L.Daily requirement of children (6 months -8 yrs.) ranging from 30 -130 mg and for adult about 400 mg.

Magnesium and health benefits[47]

Adult tissues contain about 1.04 mol (25g) of magnesium, of which 66% is located within the skeleton, 33% is intracellular, and 1% is within the extracellular compartment in our body. Although magnesium is a major constituent of bone but not a consistent component of the hydroxyapatite crystal structure. Magnesium is primarily on the crystal surface, and a portion is in equilibrium with extra cellular fluid magnesium. Magnesium is the most available divalent cation in the intracellular compartment, where it serves as a co-factor in a number of biological systems that regulate enzymatic activities and neuromuscular functions. Serum magnesium exists in the ionic state (55%), protein-bound (30%), and complexed (15%) are documented. Ionic magnesium is the fraction that most closely correlates with magnesium dependent biological actions also. Serum total or ionic magnesium is not a good estimate of intracellular, soft tissue, or total body for magnesium content.

CHAPTER THREE

METHODS AND MATERIALS

3.1 Preamble

This study was a case controlled. Because of certain logistic limitations in the laboratory at the University of Rajshahi, the laboratory work involved in this research had to be undertaken with assistance from the Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh. All sorts of administrative procedure necessary for the assisted work between two universities were maintained. The period July 2015 to July 2019 was required to complete all conventionalities from germinal to terminal end of this study. Laboratory findings of the study were noted in predefined report forms, from where they were later compiled to analyze the data using SPSS (statistical package for the social sciences) statistical software.

The study included 594 neonates were collected from the outpatient and inpatient Department of Clinical Pathology (Laboratory Medicine) and Neonatology at Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka. Infants with severe congenital malformation, sepsis, or birth asphyxia were excluded from this study. Neonates were divided into case and control groups. This study was approved by the institutional ethical committees of Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh (Approval Memo no-82/320/IAMEBBC/IBSc, 20 August, 2017).

3.2 Participants and Selection Procedure

The participants were collected from the outpatient and inpatient department of Clinical Pathology (Laboratory Medicine) and Neonatology at Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka after obtaining informed consent from neonates mother. Initially six hundred thirty eight numbers of subjects, 1-28 days aged, and both sexes were taken as cases and control. After followed the necessary inclusion and exclusion criteria, finally five hundred ninety-four numbers of patients were collected for this study.

3.2.1 Sampling Technique:

- Purposive sampling.
- Hyperbilirubinemia neonates by considering selection criteria.

Data collection instrument:

- Data collection sheet.
- Interview of mother or Legal guardian.

Selection criteria:

3.2.2 Inclusion criteria

- Neonates with both sex and age up to 28 days.
- The neonates of hyperbilirubinemia according to their specified age group were included as cases.
- The healthy neonates who had normal level of bilirubin according to their specified age group were included as controls.
- The level of serum bilirubin >140 mg/L (>14 mg/dl) in hyperbilirubinemia and <140 mg/L (<14 mg/dl) in healthy neonates according to their specified age group.

3.2.3 Exclusion criteria

- Age more than 28 days.
- Current history of illness with severe renal, respiratory and cardiovascular diseases etc.

3.3 Study Design and Grouping

This was a case control study design. After taking the written informed consent from patients initially it was divided into two groups named as group 1 (case group) and group 2 (control group). Case group and control group was contained 319 and 275 numbers of patients respectively.

3.4 Sample size calculation[48]

To calculate sample size for case control study the comparison between two means the following formula was followed:

$$\text{Sample size (n)} = \frac{(Z_\alpha+Z_\beta)^2\times(\sigma_1)^2+(\sigma_2)^2}{(\mu_1-\mu_2)^2}$$

$$= \frac{(1.96+1.64)^2\times\{(0.0036)^2+(0.002)^2\}}{(0.0021-0.0012)2}$$

n= 271.3≈ 275 in each group

Here,

μ_1 =0.0021 ppm or µg/ml or mg/L (mean zinc level in control group)

μ_2 = 0.0012 ppm or µg/ml or mg/L (mean zinc level in case group)

σ_1= .0036 ppm or µg/ml or mg/L (Standard deviation of control group)

σ_2 = .002 ppm or µg/ml or mg/L (Standard deviation of experimental group)

Zα = Z value (two tail) of standard normal distribution at 95% confidence level or 5% level of significance =1.96 (when α=0.05 at 5% level of significance)

Zβ = Z value (one tail) at 95% power =1.64 (when β=0.2 and power=1-β)

All values were taken from E. J.Hasan et. al (2011)

Four samples will be added in each group. So, target sample size will be 275 (each group) Total sample size will be 275x2=550.

3.5 Evaluation of sociodemographic profile

The sociodemographic profile includes age, sex, body weight, mother's occupation, history of ANC, socioeconomic condition was observed.

Laboratory procedure:

3.6 Blood collection and serum separation

After taking consent from mother two milliliter (2ml) blood was collected from neonate under complete aseptic conditions and was dispensed into a plain tube left to clot at room temperature (25°C) for 30 minutes, centrifuged in 3000 rpm for 5 minutes, then the serum was separated and stored in -20˚c for further test. The test was done in few successive occasion, one week apart.

Fig 3.1: Blood sample collection procedure

Fig 3.2: Centrifuge machine with micropipette

3.7 Assessment Parameters

The level of bilirubin and magnesium was assessed in automated methods (Siemens Dimension RxL Max, USA) and zinc and copper was assessed in Colorimetric method (Semi-automated biochemistry analyzer, Evolution 3000, Italy) using ready for use kit. supplied by LTA s.r.l, ITALY.

Fig 3.3: Serum bilirubin and serum magnesium were measured by siemens dimension RxL Max, USA (Fully automated biochemistry analyzer)

Fig 3.4: Serum Zinc and Copper were measured by semi-automated biochemistry analyzer (Evolution-3000, Italy)

3.8 Laboratory method

3.8.1 Estimation of serum bilirubin[49]

Method: Automated photometric method (Biochemistry auto analyzer,Siemens Dimension RxL Max, USA).

Summary: There are at least four distinct bilirubin fractions that make up total bilirubin in serum. The direct reacting fractions are mono-and diconjugated bilirubin (ß and γ-bilirubin) and the delta fraction (δ-bilirubin), which is tightly bound to albumin. Unconjugated bilirubin (α-bilirubin) is water-insoluble and reacts only after addition of an accelerator such as caffeine.

Principles of procedure: Diazotized sulfanilic acid is formed by combining sodium nitrite and sulfanilic acid at low pH. Bilirubin (unconjugated) in the sample is solubilized by dilution in a mixture of caffeine/benzoate/acetate/EDTA. Upon addition of the diazotized sulfanilic acid, the solubilized bilirubin including conjugated bilirubins (mono and diglucoronides) and the delta form4 (biliprotein-bilirubin covalently bound to albumin) is converted to diazo-bilirubin, a red chromophore representing the total bilirubin which absorbs at 540 nm and is measured using a bichromatic (540, 700 nm) endpoint technique. A sample blank correction is used.

Solubilized bilirubin + Diazotized sulfanilic acid ⟶ Red chromophore (absorbs at 540 nm)

Reagents:

Wells	Form	Ingredients	Concentration
1, 4-6	Liquid	Acetate Buffer Caffeine	337 mM
		Sodium Benzoate	168 mM
		Disodium EDTA	2.57 mM
2	Liquid	Sulfanilic acid	25.89 mM
		Hydrochloric acid	132 mM
3	Liquid	Sodium Nitrite	72.5 mM

Sample collection and handling: Serum, lithium heparin plasma, and EDTA plasma can be collected by normal procedures. Serum and plasma specimens should be separated from cells within 2 hours after venipuncture. Specimens should be free of particulate matter. To prevent the appearance of fibrin in serum samples, complete clot formation should take place before centrifugation. Clotting time may be increased due to thrombolytic or anticoagulant therapy. Bilirubin is photosensitive. Care should be taken to protect sample from both daylight and fluorescent light to avoid photodegradation. Separated specimens are stable for 8 hours at room temperature, 7 days at 2 – 8 ºC or 6 months frozen at 20 °C or colder. Protection from light is required when specimens are stored for more than 8 hours.

Materials & equipments

1. Automated Biochemistry analyzer (Siemens Dimension RxL Max, USA) or (Semi-automated biochemistry analyzer, Evolution 3000, Italy) or Spectrophotometer
2. Test tubes with rack
3. Controls and calibrators
4. Timer
5. Distilled water

Test procedure (Bilirubin)

Sampling, [c]reagent delivery, mixing, processing and printing of results are automatically performed by the Dimension system.

c. The sample container must contain sufficient quantity to accommodate the sample volume plus dead volume.

Test conditions

Sample	10 µL
Reagent 1	250 uL
Reagent 2	47 uL
Temperature	37 °C
Wavelength	540, 700 nm

Calibration

Assay range	0.1 – 25.0 mg/dL [2 – 428 µmol/L]d
Calibration material	TBI/DBI Calibrator, Cat. No. DC167
Calibration scheme	3 levels, n = 3
Units	mg/dL [µmol/L]
	(mg/dL x 17.1) = [µmol/L]
Typical calibration Levels	0.00, 10.00, 25.00 mg/dL**,**
	0, 171, 428 µmol/L
Calibration frequency	Every 90 days for any one lot

Quality control:

At least once each day of use, analyze two levels of a quality Control (QC) material with known total bilirubin concentrations.

Reference range[50]

Serum/Plasma	r**ange (mg/dL)**	**range (µmol/L)**
Premature newborn		
Up to 24 hours	1.0 to 8.0	17 to 137
Up to 48 hours	6.0 to 12. 0	103 to 205
3 to 5 days	10.0 to 14.0	171 to 239
Full-term newborn		
Up to 24 hours	2.0 to 6.0	34 to 103
Up to 48 hours	6.0 to 10.0	103 to 171
3 to 5 days	4.0 to 8.0	68 to 137

Linearity:

The method of Bilirubin is linear up to 25 mg/dl (428 µmol/L).

3.8.2 Estimation of serum magnesium[49]

Method: Automated photometric method (Biochemistry auto analyzer,Siemens Dimension RxL Max, USA).

Summary: The magnesium method is a modification of the methylthymol blue (MTB) complexometric procedure described by Connerty, Lau, and Briggs. The barium salt of ethylenebis (oxyethylenenitrilo) tetraacetic acid (Ba-EGTA) is used to reduce interference due to calcium which also reacts with methylthymol blue.

Principles of procedure: methylthymol blue (MTB) forms a blue complex with magnesium. Calcium interference is minimized by forming a complex between calcium and Ba-EGTA (chelating agent). The amount of MG-MTB complex formed is proportional to the magnesium concentration and is measured using a bichromatic (600 and 510 nm) endpoint technique.

Mg^{++} + MTB $\longrightarrow$ Mg-MTB complex (absorbs at 600 nm)

Ca^{++} + Ba-EGTA $\longrightarrow$ Complex (nonabsorbing at 600 nm)

Reagents :

Wells	Form	Ingredients	Concentration
1-3	Liquid	MTB	0.0528 g/L
		Acetic acid	
		Potassium Sorbate	
4-6	Liquid	Ba-EGTA	0.5 mM
		Sodium metaborate Buffer	
		Microbial inhibitors	

Specimen collection and handling: Normal procedures for collecting serum, plasma and urine may be used for samples to be analyzed by this method. Complete clot formation should take place before centrifugation. Separated specimens are stable for 7 days at room temperature, 7 days at 2 – 8 °C. For longer storage, specimens may be frozen at -20 °C or colder for up to a year.

Materials & equipments:

1. Automated biochemistry analyzer (Siemens Dimension RxL Max, USA) or (Semi-automated biochemistry analyzer, Evolution 3000, Italy) or Spectrophotometer
2. Test tubes and rack
3 Controls and calibrators
4. Timer
5. Distilled water

Test procedure (Magnesium)

- Sampling reagent delivery, mixing, processing, and printing of results are automatically performed by the dimension system.
- The sample container must contain sufficient quantity to accommodate the sample volume plus quiet volume.

Test conditions

Sample	4 µL
Reagent 1	100 µL
Reagent 2	200 µL
Diluent	696 µL
Temperature	37 °C
Wavelength	600 and 510 nm
Type of Measurement	Bichromatic endpoint

Calibration:

Assay range	0.0 – 20.0 mg/dl [0.0 – 8.22 mmol/L]
Calibration material	CHEM II Calibrator, Cat. No. DC20
Calibration scheme	3 levels, n = 3
Typical calibration levels	0.0, 9.0, 18.0 mg/dl, 0.0,3.70,7.41 mmol/L
Calibration frequency	Every 3 months for any one lot

Quality control:

Follow government regulations or accreditation requirements for quality control frequency. At least once each day of use, analyze two levels of a quality Control (QC) material with known magnesium concentrations.

Reference range[50]

Serum/plasma	**range (mg/dL)**	**range (mmol/L)**
Newborn, 2 to 4 days	1.5 to 2.2	0.62 to 0.91
5 months to 6 years	1.7 to 2.3	0.70 to 0.95
6 to 12 years	1.7 to 2.1	0.70 to 0.86
12 to 20 years	1.7 to 2.2	0.70 to 0.91
Adult	1.6 to 2.6	0.66 to 1.07

Linearity: The method of magnesium is linear up to 20 mg/dl (8.22 mmol/L)

Serum bilirubin and serum magnesium was measured by automated biochemistry analyzer in the Department of Laboratory Medicine Bangabandhu Sheikh Mujib Medical University (BSMMU),Dhaka, Bangladesh.

3.8.3 Estimation of serum copper[51]

Method: Colorimetric method (Semi-automated biochemistry analyzer, Evolution 3000, Italy)

Principle of the assay: At pH 4.7, copper forms with 4-(3,5-dibromo-2- pyridylazo)-N-ethyl-sulfopropyl-aniline a chelate complex. The increase of absorbance of this complex can be measured and is proportional to the concentration of total copper in the sample.

$$\text{Copper} + \text{Di-Br-PAESA} \xrightarrow[\text{Medium}]{\text{Acidic}} \text{Colored complex}$$

Specimen collection: Serum or plasma not haemolyzed. Use of anticoagulant heparin salt.

Reagents:

Reagents A : Acetate buffer 0.1 M

pH : 4.9 ; reducing agents and preservatives.

Reagents B : 3,5 Di-Br-PAESA (4- (3,5-dibromo-2-pyridylazo)-Nethyl-N sulfopropylaniline) ;preservatives

Standard : Copper 200 µg/dl

Materials & equipments:

1. Normal laboratory equipment.
2. Biochemistry analyzer
3. Micropipette (20 µl,50 µl, 100 µl,1000 µl)
4. Cuvette
5. Distilled water.

Working Reagents preparation: Equal volume of the Reagent A with Reagent B mixing slowly. Reagents are stored at 2-8°C and are stable until expiration date on label.

Test procedure:

Kind of analysis: End point

Result time : 10 minutes

Colour stability: 30 minutes

Wavelength : 580 nm (570-590)

Temperature : 20-25°C

Lightpath : 1 cm

Zero : Blank Reagent

Procedure: (Serum copper)

Bring all reagents and samples to room temperature.

Pipette into test tubes	Blank	Standard	Sample
Working reagent	1000 µl	1000 µl	1000 µl
Dist. water	66 µl	-	-
Sample	-	-	66 µl
Standard	-	66 µl	

Mix and wait for 10 minutes then read the absorbances against the blank at 580 nm.

Calculation:

$$\text{Copper } \mu g/dl = \frac{A \text{ (sample)}}{A \text{ (standard)}} \times 200 \text{ (Concentration of standard)}$$

$$\text{Copper } \mu mol/L = \frac{A \text{ (sample)}}{A \text{ (standard)}} \times 31.4 \text{ (Concentration of standard)}$$

Reference range[52]

Newborns 12-67 µg/dl (1.89-10.54 µmol/L)

Adult male 80-140 µg/dl (12.59-22.03 µmol/L)

Adult female 80-155 µg/dl (12.59-24.39 µmol/L)

Linearity:

This procedure is linear up to 500 µg/dl (78.68 µmol/L).Values exceed this limit,dilute the sample with distilled water and repeat the assay. Calculation the value using the proper dilution factor.

3.8.4 Estimation of serum zinc[53]

Method: Colorimetric method (Semi-automated biochemistry analyzer, Evolution 3000, Italy)

Principle of the assay: The Zinc produces with 5-Br-PAPS[(2-5-Brom-2-pyridylazo)- 5-(N-propyl-N-sulfopropyl-amino)-phenol] a stable coloured complex which colour intensity is directly proportional to the amount of zinc in the sample. The interferences due to the oligoelements (iron,copper,cobalt) present in the sample, are eliminated using specific masking agents.

$$\text{Zinc} + \text{5-Br-PAPS} \xrightarrow[\text{Medium}]{\text{Acidic}} \text{Colored complex}$$

Specimen collection: Serum or plasma not haemolyzed. Use only heparin salt as anticoagulant.

Reagents:

Reagent A:	Borate buffer 0.37 M
pH :	8.2; Saliciladoxime 12 mM; Dimetilgioxime 1.25 mM; surfactants and preservatives.
Reagent B:	NITRO-PAPS; 0.4 mM, preservatives
Standard:	Zinc ion 200 µg/dl (30.6µmol/l);

Materials & equipments :

1.Normal laboratory equipment.

2.Biochemistry analyzer or spectrophotometer

3.Micropipette.(50 µl,100 µl, 1000 µl)

5. Test reading in cuvette .

6.Distilled water

Working Reagents preparation : Add 2000 µl of reagent B to a bottle of reagent A. Work Reagent is stable 15 days at 2-8°C.

Test procedure:

Assay tipe	:	Endpoint
Incubation time	:	10 minutes
Colour stability	:	15 minutes
Wavelength	:	560 nm (520-570)
Temperature	:	20-25°C
Light path	:	1 cm
Reading	:	Against blank reagent

Procedure: (Serum zinc)

Pipette into test tubes	Blank	Standard	Sample
Working reagent	1000 µl	1000 µl	1000 µl
Sample	-	-	50 µl
Standard	-	50 µl	-
Dist.water	50 µl	-	-

Mix well and incubate at room temperature for 10 minutes. Then measure the absorbance of the standard and test sample against the blank within 30 minutes by Semi-automated biochemistry analyzer, (Evolution 3000, Italy).

Calculation:

Zinc µg/dl = $[A_{(sample)} / A_{(standard)}] \times 200$ (Concentration of standard)

Zinc µmol/l = $[A_{(sample)} / A_{(standard)}] \times 30.6$ (Concentration of standard)

Reference range[52]

Baby/Neonate: 49.5-99.7 µg/dl (7.6-15.3 µmol/L)

Adult male: 72.6-127 µg/dl (11.1-19.5 µmol/L)

Adult female: 70-114 µg/dl (10.7-17.5 µmol/L)

Linearity: The method of zinc is linear up to 1000 µg/dl.(152.95 µmol/L)

Serum copper and serum zinc was measured by semi automated biochemistry analyzer in the Department of Laboratory Medicine Bangabandhu Sheikh Mujib Medical University (BSMMU), Bangladesh

Quality control:

1. Check instruments and light sources
2. Check cleanliness of all equipments in use
3. Check reaction temperature
4. Check expiry date of kit and contents
5. Check quality control result.

Study procedure:

3.9. Ethical consideration:

The study protocol was submitted to the Institutional Ethics Committee (IEC) before starting the work. According to the instruction of IEC, every procedure was maintained all through the study. Particularly consent, sensitive issue, confidentiality, privacy and safety of the subjects was protected during the processes.

3.10. Data collection procedure

The demographic data was collected through individual in-depth interviews, with questionnaires and followed particular assessment criteria and data of age, sex, body weight, mothers occupational and history of antenetal care (ANC) was recorded. Blood samples for biochemical examinations were collected directly from the vein of each patient. After collection blood samples, standard procedure was followed to evaluate all the investigations and report was collected in a hospital based format. The collected data was documented in some particular pro-forma (see in appendix E) and then immediately it was computed.

3.11 Data processing and Statistical analysis

Statistical analysis was done by using the SPSS (statistical package for the social sciences) statistical software to obtain the mean, standard deviation and frequencies. The following statistical tests were used:

a. Mean and standard deviation (SD) to describe quantitative data.

b. Student t test was used to compare between two groups.

c. Chi-square test was used to compare between two groups.

d. Pearson correlation was used to correlate two quantitative variables. All tests, a probability (p) of <0.05 was considered significant.

3.12. Data presentation

Data and results were presented in the form of tables and diagram where applicable.

3.13 Materials required

3.13.1 Collection and preservation of chemicals and reagents

The various chemicals and reagents used in the experiment were prepared, collected and preserved as appropriate.

3.13.2 Regents for biochemistry: Regents kits were collected from LTA s.r.l and biomed diagnostics Laboratory, Siemens Healthcare Diagnostics Ltd
and preserved in refrigerator.

3.13.3 Instrument and appliances (Raanco, Sialkot, Pakistan): These are

- Ordinary forceps
- Towel and clips
- Sterile cottons 500 gm pack (Sun-moon corporation, Bangladesh)
- Digital weight machine
- Colorimeter and cuvette
- Centrifuge machine
- Microscope (Spencer, USA)
- Beakers 50, 100, 250, 500, 1000 ml
- Eppendorf (plastic)
- Clean glass slide
- Incubator
- Refrigerator: Normal refrigerator (Nikkon, India), -20°C freezer (PR-100 F, Cincinnati, Ohio, USA)
- pH meter (C, G 840, UK)
- Micropipette with tips: Air displacement adjustable volume pipette (Sigma, USA), 100 ml capacity, yellow colour tips for air displacement adjustable volume pipette (Sigma, USA)
- Disposable plastic syringes (10 and 5 ml; Kana Korporation, Korea)
- Ethylenediaminetetraacetic acid (EDTA) vial
- Staining rack (wooden)
- Labeled test tubes or red vial
- Glass slides 70 × 26 mm (Chance Proper Ltd)
- Cover slips
- Sterile cotton 500 gm pack (Sun-moon Corporation, Bangladesh)
- Plastic containers
- Polythene bag (size 14 × 100 cm) 500 gm

3.14 Quality control

The study was conducted under close supervision of the research supervisors who alerted and guided the research through potential threats. Standard equipment and materials were used. Standard laboratory procedures were followed. The results of laboratory examinations were recorded in a predefined format so that no data was lost. Data was carefully entered into computer which was concurrently and retrospectively checked to prevent ‘duplicate entry’, ‘no entry’ or any other inconsistency.

CHAPTER FOUR

RESULTS

The study was conducted to determine the effects of heavy metals (serum zinc, copper and magnesium) in hyperbilirubinemia and healthy neonates. Total 594 numbers of sample 319 case group and 275 control group. The sociodemographic profile includes age, sex, body weight, mothers occupational, history of antenatal care (ANC) and the levels of serum bilirubin, zinc, copper and magnesium was assessed.

4.1 Observations of demographic characteristics of all patients

4.1.1 Age group

The mean age of the respondents were 3.08 and 3.21 days regarding to 1-25 days age group in study and control groups respectively. The highest percentages of the neonates were showed in the age group of 1-2 days (55.80%) in case group and (63.64%) in control group followed by 1-25 days which is not significant (Table 4.1). In case group 1-2 days frequency is more because neonatal jaundice is mostly develop in 48 hours after birth.

Table 4.1: Demographic variable of age among case and control groups

Age variables (days)	**Case (n=319)**	**n(%)**	**Control (n=275)**	**n(%)**	**p value**
1-2	178	55.80	175	63.64	
3-7	115	36.05	70	25.45	
8-15	24	7.52	27	9.82	
Upto 25	2	0.63	3	1.09	
Mean± SD	**3.08±2.80**		**3.21±3.65**		**^{a}0.94^{ns}**

Note: s= significant, ns= not significant, ^{a}P value reached from unpaired t-test

4.1.2 Sex ratio

Table 4.2 showed that the sex variable of the respondents were male 187 and 160 in case and control group respectively and female were 132 and 115 in case and control group respectively. The highest percentage of male were 187 (58.62%) in case and 160 (58.18%) in control group followed by female 132 and 115 population both in case and control group which is not significant.

Table 4.2: Demographic variable of sex among case and control groups

Sex	**Case (n=319)**	**n(%)**	**Control (n=275)**	**n(%)**	**p value**
Male	187	58.62	160	58.18	$^{b}0.207^{ns}$
Female	132	41.38	115	41.82	

Note: s= significant, ns= not significant, ^{b}P value reached from Chi square test

4.1.3 Body weight

The mean weight of the respondents were 2.62±0.37 and 2.84±0.27 kilogram(Kg) regarding to <2.5–>3.5 kilogram(Kg) in case and control groups respectively which is showed in table 4.3. The highest percentages of the neonates were in the weight group 2.5–3.5 kilogram of 227 (71.16%) neonates in case group and 271 (98.55%) neonates in control group which is significant. Past studies also showed that neonatal jaundice is more common in low birth weight of neonates.

Table 4.3: Demographic variable of weight among case and control groups

Weight (kg)	**Case (n=319)**	**n(%)**	**Control (n=275)**	**n(%)**	**p value**
<2.5	90	28.21	2	0.73	$^{a}0.001^{s}$
2.5-3.5	227	71.16	271	98.55	
>3.5	2	0.63	2	0.73	
Mean±SD	**2.62±0.37**		**2.84±0.27**		

Note: s= significant; ns= not significant, ^{a}P value reached from unpaired t-test

4.1.4 Mother's occupation

Table 4.4 showed that the housewives were 240 and 218; service holders were 58 and 40 and businesses were 21 and 17 numbers of mother's occupation in case and control group respectively. The highest percentage of mother's occupation were housewife 240 (75.23%) in case and 218 (79.27%) in control group followed both in case and control group which is not significant.

Table 4.4: Demographic variable of mother's occupation among case and control groups

Mother's Occupation	Case (n=319)	%	Control (n=275)	%	Total	p value
House wife	240	75.23	218	79.27	496	0.23^{ns}
Service	58	18.18	40	14.55	98	
Business	21	6.58	17	6.18	38	
Total	**319**		**275**		**594**	

Note: s= significant; ns= not significant

4.1.5 Antenatal checkup

Table 4.5 showed that the antenatal checkup variable of mothers were regular checkup 301 (94.36%) and 258 (98.82) in case and control group respectively and irregular checkup were 18 and 17 in case and control group respectively and which is not significant.

Table 4.5: Demographic variable of antenatal checkup among case and control groups

ANC	Case (n=319)	n (%)	Control (n=275)	n (%)	Total	p value
Regular	301	94.36	258	98.82	559	0.23^{ns}
Irregular	18	5.64	17	6.18	35	

Note: s= significant; ns= not significant

4.1.6 Socioeconomic status

Table 4.6 showed that the highest socioeconomic statuses of mothers were 225 and 173 in case and control group respectively and those are come from upper middle class family.

Table 4.6: Demographic variable of socioeconomic status of mothers among case and control groups

Socio-economic status	Case (n=319)	Control (n=275)	Total	p value
< 10000 (Lower)	07	18	25	0.007[s]
10001-20000 (Lower middle)	58	67	125	
20001-40000 (Upper middle)	225	173	398	
> 40000 (Upper)	29	17	46	
Total	**319**	**275**	**594**	

Note: s= significant; ns= not significant

4.2 Result of biochemical parameters

4.2.1 Serum total bilirubin

Table 4.7 showed that the concentration of serum bilirubin was 171.14 mg/L and 35.42 mg/L in case and control group respectively and which is very significant ($p<0.001$) when case group compared with control group in neonates. Case group showed serum total bilirubin is higher than control group because the liver functions become progressively worse in case group or neonatal hyperbilirubinemia group.

Table 4.7: Biochemical variable of serum total bilirubin among case and control groups

Trace Elements	Case (n=319) Mean ±SD	Control (n=275) Mean ±SD	p value
Serum total bilirubin (mg/L)	171.14 (±17.08)	35.42 (±8.38)	<0.001[s]

Note: s= significant; ns= not significant , $p<0.001$

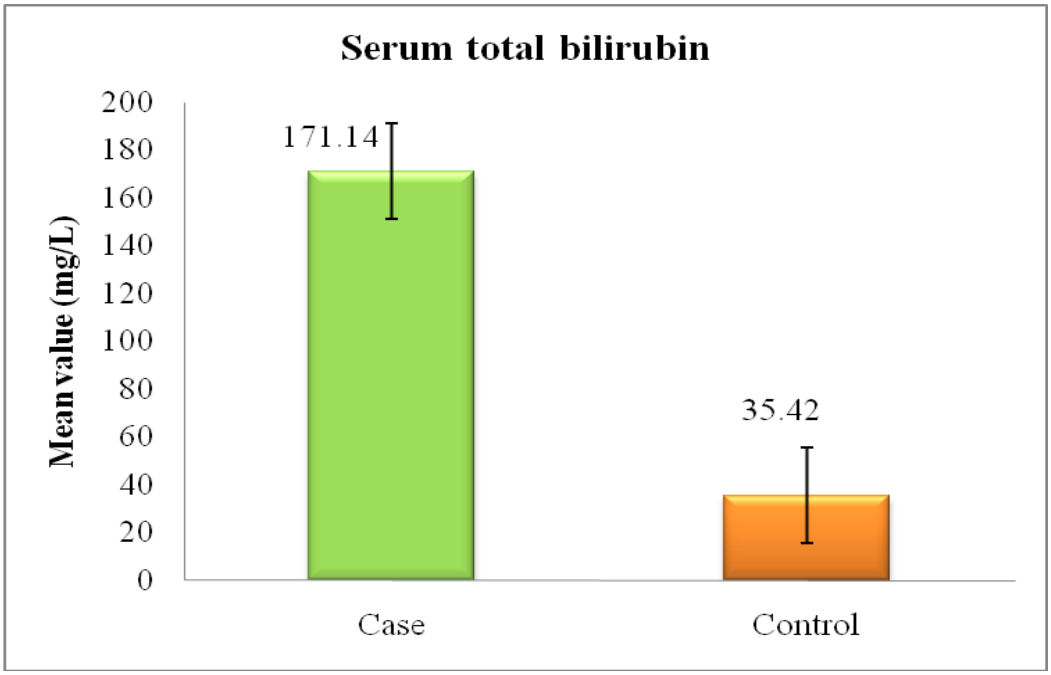

Fig. 4.1 Biochemical variable of serum total bilirubin among case and control groups

4.2.2 Serum magnesium level

The serum magnesium was 23.98 mg/L and 19.19 mg/L in case and control group respectively in table 4.8. The concentration of serum magnesium is higher in case group when is compared with control which is very significant ($p<0.001$).

Table 4. 8: Biochemical variable of serum magnesium among case and control groups

Trace Elements	Case (n=319) Mean ±SD	Control (n=275) Mean ±SD	p value
Serum magnesium (mg/L)	23.98 (±2.11)	19.19 (±1.60)	$<0.001^{s}$

Note: s= significant; ns= not significant, $p<0.001$

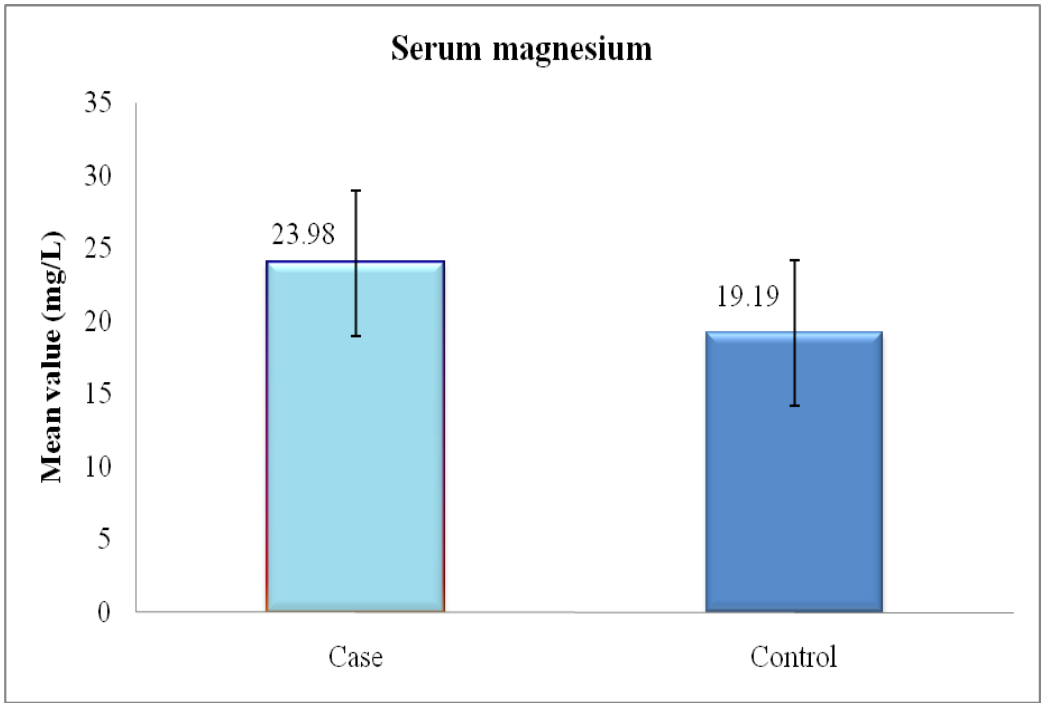

Fig 4.2: Biochemical variable of serum magnesium among case and control groups

4.2.3 Serum zinc level

In case and control group, the level of serum zinc was 0.50 mg/L and 0.68 mg/L respectively which is showed in table 4.9. The concentration of serum zinc is lower in case group when is compared with control which is very significant ($p<0.001$).

Table 4.9: Biochemical variable of serum zinc among case and control groups

Trace Elements	Case (n=319) Mean ±SD	Control (n=275) Mean ±SD	p value
Serum Zinc (mg/L)	0.50 (±0.03)	0.68 (±0.10)	<0.001[s]

Note: s= significant; ns= not significant, $p<0.001$

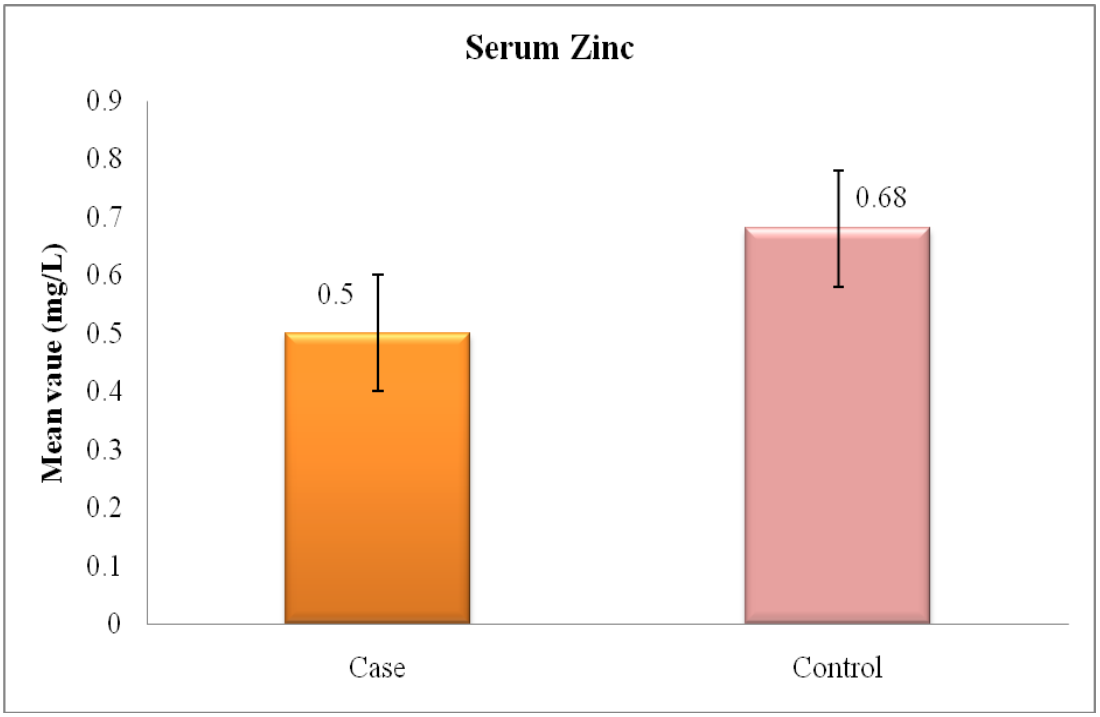

Fig. 4.3: Biochemical variable of serum zinc among case and control groups

4.2.4 Serum copper level

Table 4.10 shows the serum copper 0.75 mg/L were in case group and 0.43 mg/L were in control group. The concentration of serum copper is higher in case group when is compared with control which is very significant ($p<0.001$).

Table 4. 10: Biochemical variable of serum copper among case and control groups

Trace Elements	Case (n=319) Mean ±SD	Control (n=275) Mean ±SD	p value
Serum Copper (mg/L)	0.75 (±0.08)	0.43 (±0.11)	<0.001[s]

Note: s= significant; ns= not significant, $p<0.001$

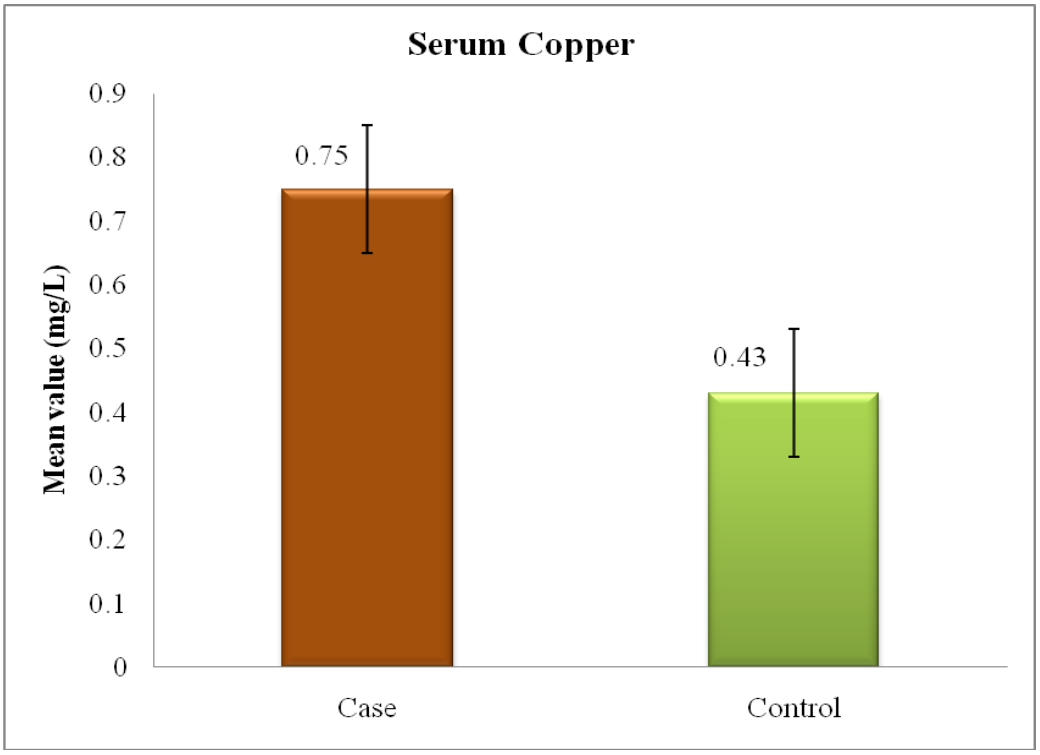

Fig. 4.4: Biochemical variable of serum copper among case and control groups

4.3 Correlation between serum total bilirubin level and serum magnesium level

The scatter diagram showing in figure 4.5 there is positive significant pearson correlation (r= 0.817, p value <0.001) between serum total bilirubin level and serum magnesium level in both groups. It means if serum bilirubin is increased, serum magnesium also increased in case group and control group.

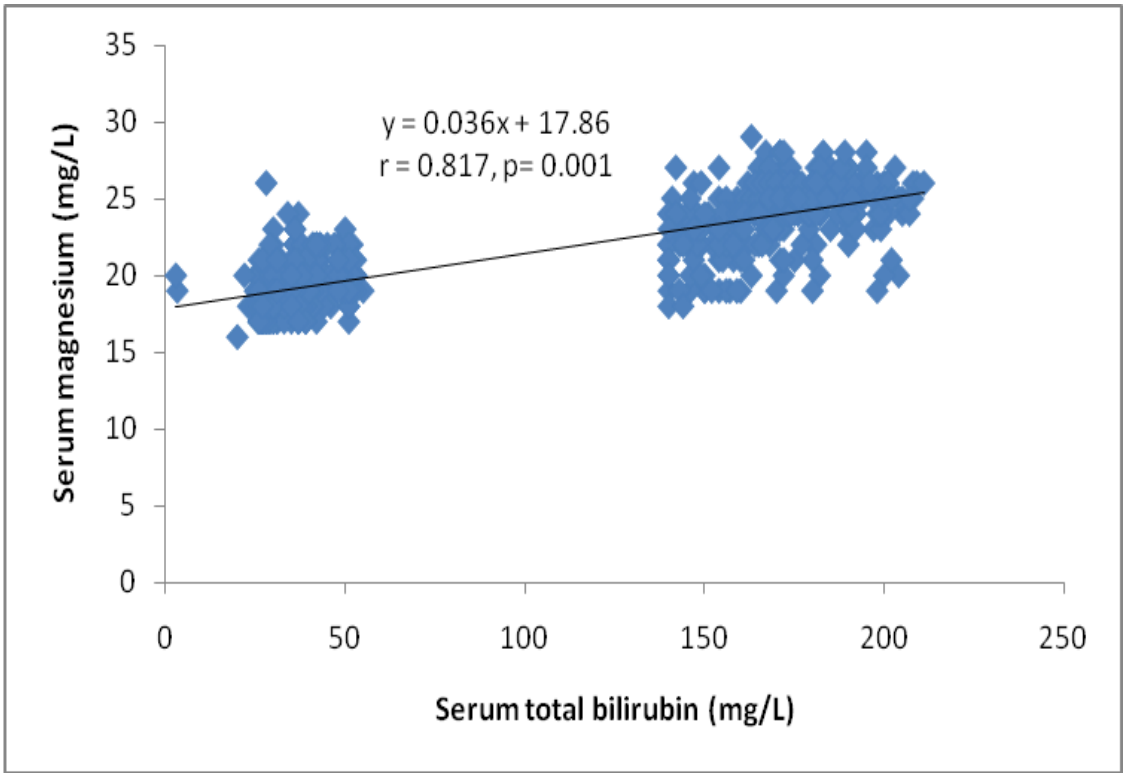

Figure 4.5: Scatter diagram of correlation between serum total bilirubin level and serum magnesium level in both groups.

4.4 Correlation between serum total bilirubin level and serum zinc level

In figure 4.6 the scatter diagram showing negative significant pearson correlation (r=-0.773, p value <0.001) between serum total bilirubin level and serum zinc level in both groups. It is justified that if serum bilirubin level is increased, the serum zinc level decreased in case group and control group.

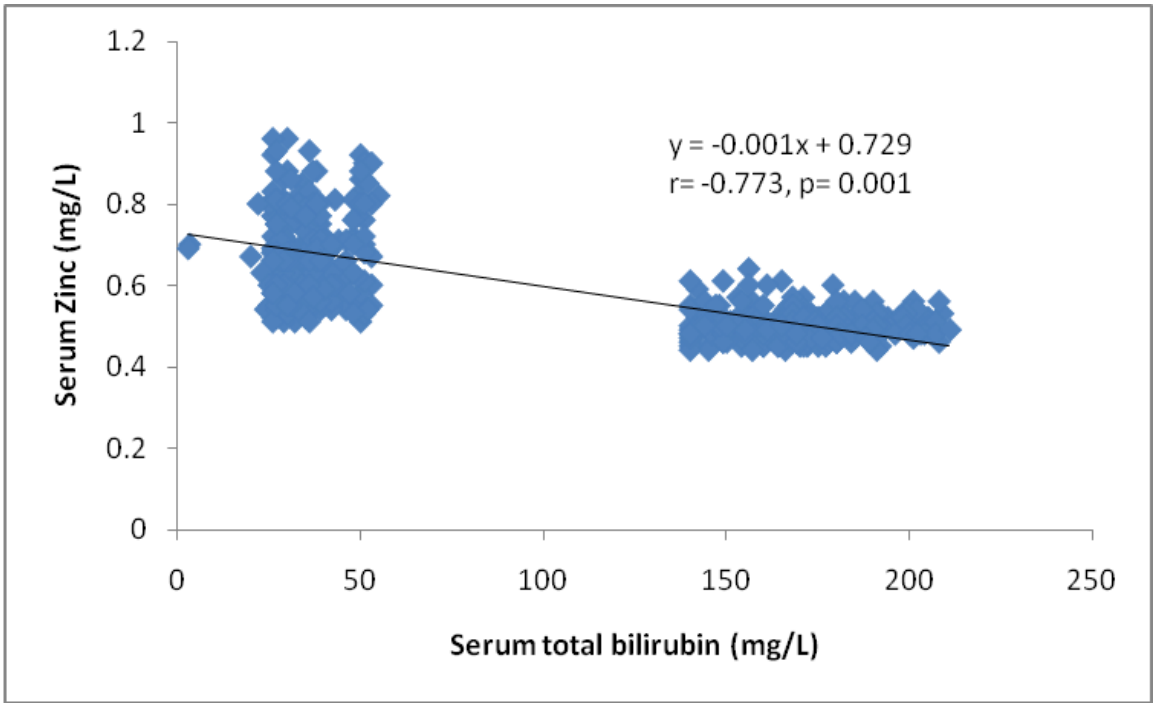

Figure 4.6: Scatter diagram of correlation between serum total bilirubin level and serum zinc level in both groups.

4.5 Correlation between serum total bilirubin level and serum copper level

Figure 4.7 the scatter diagram showing positive significant Pearson correlation (r=0.832, p value <0.001) between serum total bilirubin and serum copper level in both groups and it can be said that if serum bilirubin level is increased, the serum copper level also increased in case group and control group.

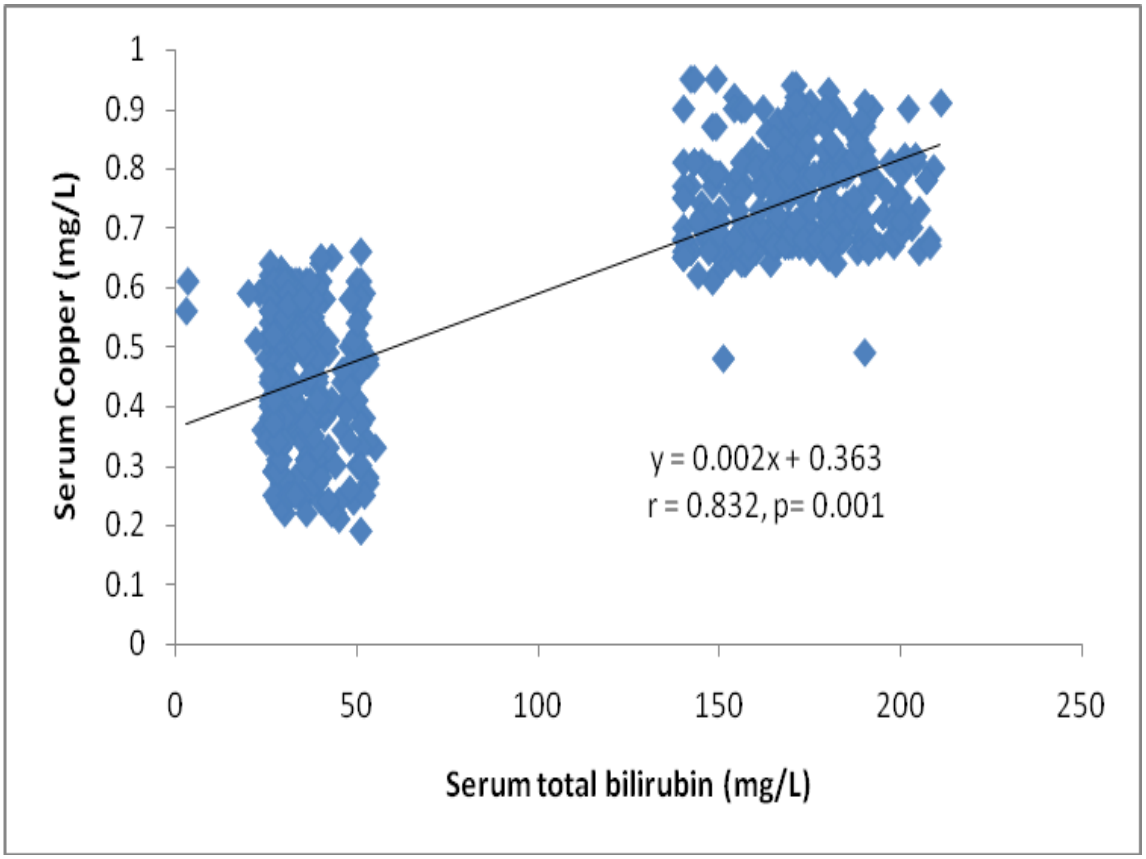

Figure 4.7: Scatter diagram of correlation between serum total bilirubin level and serum copper level in both groups.

CHAPTER FIVE

DISCUSSION

The study was conducted to determine the effects of heavy metals (serum zinc, copper and magnesium) in hyperbilirubinemic and healthy neonates. Total 594 numbers of sample 319 case group and 275 control group. The sociodemographic profile includes age, sex, body weight, mothers occupational, history of antenatal care (ANC), the levels of serum bilirubin, zinc, magnesium and copper was assessed.

5.1 Discussion of demographic characteristics

5.1.1 Age group

The mean age of the respondents was 3.08 and 3.21 days regarding to 1-25 days' age group in case and control groups respectively. The highest percentage of the neonates was showed in the age group of 1-2 days (55.80%) in case group and age group of 1-2 days (63.64%) in control group. In case group 1-2 days' frequency is more because neonatal jaundice is mostly developing in 48 hours after birth. Neonatal jaundice is a clinical condition which occurs due to accumulation of unconjugated, nonpolar, lipid-soluble bilirubin pigment as a result yellowish discoloration of the skin, sclera, and mucous membranes. In world-wide, neonatal jaundice has a significant importance in terms of neonatal morbidity and mortality.[54] Almost all newborn infants have a serum or plasma total bilirubin (TB) level >1 mg/dl in contrast to normal adults in whom the normal total bilirubin (TB) level is <1 mg/dL. Physiological jaundice usually appears on the 2^{nd} to 3^{rd} day, peaking between the 5^{th} and 7^{th} days of life. Jaundice may appear at birth or may appear any time during neonatal period depending on the cause.[55]

5.1.2 Sex ratio

The sex variable of the respondents was male 187 and 160 in case and control group respectively and female were 132 and 115 in case and control group respectively. The highest percentage of male were 187 (58.62%) in case and 160 (58.18%) in control group followed by female 132 and 115 population both in case and control group which is not significant.

Because, in human the ratio between males and females at birth is slightly biased towards the male sex. The natural "sex ratio at birth" is often considered to be around 105. This means that at birth on average, there are 105 males for every 100 females.[56] The value for sex ratio at birth (male births per female births) in Bangladesh was 1.05 as of 2015.[57] The gender ratio reffered the ratio between the number of males and females in a society. This variation of ratio depends on biological, social, technological, cultural, and economic forces.[58]

5.1.3 Body weight

The mean weight of the respondents was 2.62± 0.4 and 2.84±0.3 kilogram regarding to <2.5–>3.5 kilogram in case and control groups respectively. The highest percentage of the neonates was in the weight group 2.5-3.5 kilogram of 227 (71.16%) neonates in case group and 271 (98.55%) neonates in control group which is significant. Although 90 samples in case group showed the body weight less than 2.5 kilogram. According to a national survey, at least 22.6% of babies are born with low birth weight (below 2.5 Kg) in Bangladesh. According to the WHO, the premature birth, poor nutritional status of mother and inadequate nutritional intake during pregnancy, and intrauterine growth restriction are the major causes of low birth weight (LBW).[59] Low birth weight and premature infants are at major risk for exaggerated hyperbilirubinaemia and jaundice that can lead to bilirubin encephalopathy.[60]

5.1.4 Mother's occupation

The housewife was 240 and 218; service holder was 58 and 40 and businesses were 21 and 17 numbers of mother's occupations in case and control group respectively. The highest percentage of mother's occupation was housewife 240 (75.23%) in case and 218 (79.27%) in control group followed both in case and control group which is not significant. Understanding the nature of jaundice, early detection and bad consequences can help in protect those newborn from jaundice complication. Mothers plays a critical role in their baby life in our culture mainly the 1st day post delivery, as they responsible one for their baby care. Mothers should have enough education and awareness about early identification of jaundice signs and caring of

jaundiced baby and this will help in effective management and avoiding complications of jaundice.[61] This study revealed that most of mothers were housewife, and they were not rich in education.

5.1.5 Antenatal checkup

The antenatal checkup variable of mothers was regular checkup 301 (94.36%) and 258 (98.82%) in case and control group respectively and irregular checkup were 18 and 17 in case and control group respectively and which is not significant. Antenatal care (ANC) services are defined as an umbrella term used to describe the medical procedures and care carried out during pregnancy.[62] Antenatal care is very important to pregnant women as it helps prevent mother and child mortality, prevent complications like neonatal jaundice etc.[63] Past study showed who attended antenatal care they were aware of neonatal jaundice, and those who received educational instruction significantly had more knowledge on neonatal jaundice than those who did not receive instructions. This study revealed that antenatal care is a good souce of knowledge of the treatment and complications of neonatal jaundice but inadequate knowledge of the causes and danger signs of the condition. It is therefore that health care providers should give more health education on neonatal jaundice to the expectant mothers during antenatal visits.

5.1.6 Socioeconomic status

Table 4.6 showed that the highest socioeconomic status of mothers was 225 and 173 in case and control group respectively and those are come from upper middle class family.

5.2 Discussion of biochemical parameters

5.2.1 Serum total bilirubin

The concentration of serum bilirubin was 171.14 mg/L and 35.42 mg/L in case and control group respectively and which is very significant ($p<0.001$) when case group compared with control group in neonates. Case group showed serum total bilirubin is higher than control group because the liver functions become progressively worse in case group or neonatal hyperbilirubinemia group. Mainly bilirubin is produced from

hemoglobin catabolism, and partially from non erythroid hemeproteins, myoglobin, and ineffective erythropoiesis. The reticuloendothelial system is site of conversion of heme to bilirubin. A molecule of carbon monoxide (CO) together with bilirubin, and an atom of iron are products of the catabolism. To quantify the production of bilirubin in newborns can be he determination by the measurement of CO hemoglobin.

By the enzyme system of UDP glucuronosyltransferase (UGT) 1A1 codified by UGT 1A1 gene complex, the bilirubin is conjugated to monoconjugated and diconjugated bilirubin. When bilirubin load in newborns is high, bilirubin is mainly conjugated with only one molecule of glucuronic acid. In the intestines, monoconjugated bilirubin is more easily deconjugated by β-glucuronidase and reverted to native bilirubin which can be reabsorbed by the gut. In fact, the intestinum of neonates is almost sterile, and bilirubin is not yet degraded to stercobilinogen .[64]

Jaundice usually develops when conjugated or unconjugated bilirubin deposits in skin. Neonatal jaundice is caused by variety of physiologic and pathologic conditions. Improved bilirubin production, less competent hepatic conjugation and superior enterohepatic circulation are physiologic aspects. The pathologic aspects are two patterns of diseases such as hyperbilirubinemia and cholestassis.
Isoimmune hemolytic disease is the most important maternal effect on neonatal hyperbilirubinemia. ABO incompatibility is the most common cause of isoimmune hemolytic disease.[65]

5.2.2 Serum magnesium level

The serum magnesium was 23.98 mg/L and 19.19 mg/L in case and control group respectively. The concentration of serum magnesium is higher in case group when is compared with control which is very significant ($p<0.001$). The serum levels of magnesium become high, depending on the existence of mild hemolysis in newborn in case group. Magnesium is a cofactor in multiple enzymatic reactions, including those involving energy metabolism as well as DNA and protein synthesis, and it participates in the regulation of ion channels. So, the magnesium homeostasis is

fundamental to the existence of life.[66] Magnesium is found almost entirely in the intracellular compartment. The small serum component gives a poor representation of the active, physiologic state of the metal. The increase magnesium level in case group may be due to extracellular movement of intracellular magnesium because of cellular injury by high bilirubin level that may cause neuronal and generalized cellular injury.[67] Another study also reported that reported that the increment in plasma magnesium may be due to release of intracellular magnesium result from cellular injury of erythrocytes. This may be due to increment of red cell mass (RBCs volume per kilo in neonate) and due to decrement of fetal RBCs life span, resulting in earlier destruction and hemolysis of these fetal RBCs and subsequently increment in serum unconjugated bilirubin mainly and serum ionized magnesium.[68] More study found that increases in plasma ionized magnesium may be due to extracellular movement of intracellular magnesium resulting from cellular injury of neurons and erythrocytes. Indeed, bilirubin toxicity after the increase of serum bilirubin values to toxic levels not only is limited to neurons but also may cause generalized cellular injury.[69] Other study also showed that increase in plasma ionized magnesium may be due to extracellular movement of magnesium, a principally intracellular ion, resulting from generalized cellular injury including neurons and erythrocytes. This increase has neuroprotective role against emerging toxicity risk of increasing serum bilirubin levels.[43]

5.2.3 Serum zinc level

In case and control group, the level of serum zinc was 0.50 mg/L and 0.68 respectively. The concentration of serum zinc is lower in case group when is compared with control which is very significant ($p<0.001$). For the development of human body zinc is an essential element. Zinc plays a significant role in cellular cohesion, natural growth and development, proper performance of immune system and improves appetite, and is necessary for many vital functions of body.

In pregnant mother, the level of zinc also performances an important role in fetal-neonetal outcomes like growth, birth weight, neurobehavioral development, performance of immune system. Adequate amount of zinc in pregnant women is

essential for the optimum health of mother, embryo and neonatal, and reduction of mortality rate.[9]

Zinc is a vital element in human nutrition with a wide range of biological functions. It strengthens the physical growth of digestive and immune systems as well as decreasing the infection rate. The stunted growth, increased incidence of infections like pneumonia, gastroenteritis and neurobehavioral change in infants and children may cause zinc deficiency.[70]

The concentration of zinc is lower in case group because zinc plays a role in decreasing serum bilirubin by inhibiting its enterohepatic circulation (EHC) of bilirubin via enhancement of bilirubin sequestration or degradation in the intestinal lumen. Oral zinc presumably reduces the serum total bilirubin by precipitating unconjugated bilirubin (UCB) from unsaturated micellar solution of bile salts consequently inhibiting the EHC of bilirubin, possibility of zinc as an agent in the treatment of severe unconjugated hyperbilirubinemia and also in the prevention of its development in at-risk neonates, appears to be an attractive low cost, low risk intervention.[71]

5.2.4 Serum copper level:

The serum coppers 0.75 mg/L were in case group and 0.43 mg/L were in control group. The concentration of serum copper is higher in case group when is compared with control which is very significant ($p<0.001$). Past study showed that the elevated level of serum copper is documented in liver disease like cirrhosis, obstructive jaundice and cholestasis. In intentional or accidental ingestion and hemolysis, such as glucose-6-phosphate dehydrogenase deficiency hemolysis, results increase nonceruloplasmin copper in plasma due to copper overload. Copper also plays an essential role in mitochondrial electron transport and activate the two enzymae intracellular copper zinc superoxide dismutase and cytochrome oxidase that work as antioxidant.[72]

Copper is an essential trace element for humans. In almost every cell of the human body copper can be found. The maximum concentrations of copper are exposed in the brain and the liver; the central nervous system and heart. In bones and muscles about 50% of copper content can be stored (in skeletal muscle it is about 25%), 15% in skin, 15% in bone marrow, 8 to 15% in the liver and 8% in the brain.

Several essential enzymes, such as copper enzymes cytochrome c oxidase, lysyl oxidase, feroxidase, 2-furoate-CoA dehydrogenase, amine oxidase, catechol oxidase, tyrosinase, dopamine beta-monooxygenase, D-galaktozo oxidase, D-hexozo oxidoreductase, indole 2.3- dioxygenase, L-ascorbatoxidase, nitratreductase, peptidylglycine monooxygenase, flavonol 2,4-dioxygenase, superoxide dismutase, PHM (peptidylglycine monooxygenase hydroxylation) and others are functional component of copper.[73]

Copper is an is an important cofactor for many proteins because its an essential tarce element. The recommended intake is 0.9 mg/day and, typically 2-5 mg/day. Usually copper is absorserd mainly in the duodenum and proximal small intestine by enterocytes through transported in the portal circulation in association with albumin and the amino acid histidine to the liver then avidly removed from circulation. Most of dietary copper excreate through renal system. For metabolic needs, the liver utilizes some copper for metabolic needs, synthesizes and secretes the copper-containing protein ceruloplasmin, and excretes excess copper into bile. So in neonatal jaundice, the processes of biliary copper excretion can be impairing and that may lead to increases in hepatic copper content.[74] Copper destroys the free radicals and prevents cellular damage due to its antioxidant activity. It helps to maintain a lots of unction of human body including glucose oxidation, absorb iron, ssupply oxygen to body tissues, blance hormonal secretion etc. So it is a necessary and desirable element for the growth and development of infants.[17]

5.3 Correlation between serum magnesium, zinc, copper with total bilirubin

5.3.1 Correlation between serum total bilirubin level and serum magnesium level

This study showed there is positive significant pearson correlation ($r=0.817$, $p<0.001$) between serum total bilirubin level and serum magnesium level in both groups. It means if serum bilirubin is increased, serum magnesium also increased in case group and control group. It may be due to extracellular movement of magnesium, resulting from generalized cellular injury including neurons and erythrocytes in plasma ionized magnesium increase against emerging toxicity risk of increasing serum bilirubin values.[43]

5.3.2 Correlation between serum total bilirubin level and serum zinc level

Total bilirubin level and serum zinc level showed in both groups the negative significant pearson correlation ($r = -0.773$, $p<0.001$) between serum total bilirubin level and serum zinc level in both group. It is justified that if serum bilirubin level is increased, the serum zinc level is decreased in case group and control group. Because lower level of zinc inhibits the enterohepatic circulation.

5.3.3 Correlation between serum total bilirubin level and serum copper level

Figure 4.7 the scatter diagram showing positive significant pearson correlation ($r=0.832$, $p<0.001$) between serum total bilirubin and serum copper level in both groups and it can be said that if serum bilirubin level is increased, the serum copper level is also increased in case group and control group. The level of copper has a strong correlation with the normal functioning of red blood cell. So the liver can be dysfuction by excess overload by copper. As a result, it may cause the haemolysis. High copper concentrations of copper can occur secondarily to liver disease that prevents normal excretion of copper through the biliary system and it is probably based on free radical-induced damage to lipid membranes.[75]

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion:

It can be concluded that current study showed deficiency of zinc (Zn) followed by Jaundice may play a role of neonatal hyperbilirubinemia. The concentrations of magnesium (Mg) and copper (Cu) levels were found to be significantly greater than control groups. Therefore, Magnesium, Zinc and Copper may have relationship with Jaundice for neonates.

6.2 Recommendation:

This work has done with many limitations. So future study should consider the following points before starting the work.

- The future study can be done in multicenter study in rural and urban or slum area of Bangladesh along with more sample size.
- Trace elements selenium (Se), chromium (Cr), cobalt (Co), iodine (I), manganese (Mn), and molybdenum (Mo) should be taken in further study.
- Some others history like dietary, family history, drug history of mother should be included in future study.
- Molicular study can be done in hyperbilirubinemia patient in newborn.

6.3 Limitation and delimitation:

The study was done in Dhaka city so, it not the real picture of overall of Bangladesh. Due to limitation of budget only three trace elements was undertaken. Some other history like dietary history, drug history of mother and others history was not taken due to shortage of time.

6.4 Summary:

This study was carried out in the period January 2015 to January 2019. The study included 594 neonates were collected from the outpatient and inpatient Department of Clinical Pathology (Laboratory medicine) and Neonatology at Bangabandhu

Sheikh Mujib Medical University (BSMMU), Dhaka. Infants with severe congenital malformation, sepsis, or birth asphyxia were excluded from this study. Neonates were divided into case and control groups. This study was approved by the institutional ethical committees of Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh (Approval Memo no-82/320/IAMEBBC/IBSc, 20 August, 2017).

The mean age of the respondents was 3.08 and 3.21 days; the sex variable of the respondents were male 187 and 160 in case and control group respectively and female were 132 and 115 in case and control group respectively which was not significant. The mean weight of the respondents was 2.62±0.4 and 2.84±0.3 kilogram (Kg) which was significant. There were no significant changes in mother's occupation, antenatal checkup and socioeconomic status.

The concentration of serum bilirubin was 171.14 mg/L and 35.42 mg/L in case and control group respectively and which is very significant ($p<0.001$) when case group compared with control group in neonates. Case group showed serum total bilirubin is higher than control group because the liver function becomes progressively worse in case group or neonatal hyperbilirubinemia group. The serum magnesium was 23.98 mg/L and 19.19 mg/L in case and control group respectively. The concentration of serum magnesium is higher in case group when is compared with control which is very significant ($p<0.001$). In case and control group, the level of serum zinc was 0.50 mg/L and 0.68 mg/L respectively and the concentration of serum zinc is lower in case group when is compared with control which is very significant ($p<0.001$).

It may be result in deficient synthesis of assorted enzymes that play a role in the bilirubin metabolism and also cause structural defects in the erythrocyte membranes, resulting in hemolysis. The concentration of serum copper is higher in case group when is compared with control which is very significant ($p<0.001$). It may be due to liver disease like cirrhosis, obstructive jaundice and cholestasis. There was a positive significant pearson correlation ($r=0.817, p<0.001$) between serum total bilirubin level and serum magnesium level in both groups. It means if serum bilirubin is increased, serum magnesium is also increased in case group and control group. Relation

between serum total bilirubin level and serum zinc level was negative significant pearson correlation ($r=-0.773$, $p<0.001$) in both groups. It is justified that if serum bilirubin level is increased the serum zinc level is also decreased in case group and control group. The positive significant pearson correlation ($r=0.832$, $p<0.001$) was in between serum total bilirubin and serum copper level in both groups and it can be said that if serum bilirubin level is increased, the serum copper level also increased in case group and control group.

So in this study, the heavy metal like zinc, copper and magnesium has relation with neonatal hyperbilirubinemia. If serum bilirubin is increased, serum magnesium and copper also increased but when serum bilirubin level is increased, the serum zinc level decreased in the patient of neonatal hyperbilirubinemia. This study was also the first time recorded in Bangladesh.

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APPENDIX A

WORK SCHEDULE

*Main task	Indicative period (year)								
	2015	2016		2017		2018		2019	
	2nd half	1st half	2nd half	1st half	2nd half	1st half	2nd half	1st half	2nd half
Admission formalities for MPhil, and contact with experts in the field of study	—								
Consultation of recorded communication	—	—	—	—	—	—	—	—	
Preparation of draft research proposal and instrument	—								
Site preparation for laboratory work at BSMMU		—							
Finalization of research proposal and instrument		—							
Laboratory work and data collection		—	—	—	—				
Preliminary report on work done and presentation in a seminar at the RU						—			
Adjustment of suggestions from the seminar, and formalities for conversion to PhD program						—			
Extended laboratory work and data collection							—		
Data compilation and analysis								—	
Chapter-wise write up of draft dissertation								—	
Presentation of draft dissertation in a final seminar at the RU, and other official formalities									—
Finalization of the dissertation and submission to the RU									—

*****At every steps of the above tasks, the Research Supervisor and/or Co-supervisor were consulted

Bangla to English version

APPENDIX B

ACCEPTANCE LETTER FOR DOCTORAL PROGRAM AT THE UNIVERSITY OF RAJSHAHI
Institute of Biological Sciences
University of Rajshahi

Memo no.: 275/M-515 (15–16)/IBSc Date: 03 May 2018

Mohammad Anwar Hossain
MPhil Fellow
Session 2015-16, Reg. No-0122
Institute of Biological Sciences
University of Rajshahi, Bangladesh

Subject: Regarding conversion from MPhil to PhD program
Dear Sir:
In response to your application dated 11 March 2018, your MPhil program has been converted to PhD program.
Research title: ‘‘Correlation between heavy metals and neonatal hyperbilirubinemia among the patients attended at Bangabandhu Sheikh Mujib Medical University’
Approved in the meetings of–

Academic Committee:	222th meeting,	Dated 14 May 2018,	Decision no. 6
Board of Studies:	90th meeting,	Dated 15 March 2018,	Decision no. 14
Board of Governors:	98nd meeting,	Dated 25 March 2018,	Decision no. 12
Academic Council:	246th meeting,	Dated 05 April 2018,	Decision no. 40
Syndicate:	478th meeting,	Dated 07 April 2018,	Decision no. 02

Thanking You

(Signature: Illegible)
03 May 2018
Secretary
Institute of Biological Sciences
University of Rajshahi
Copy to:
Prof. Dr.Md. Ekramul Islam, Professor, Department of Pharmacy, University of Rajshahi
Prof.Dr. Ashik Mosaddik, Professor, Department of Pharmacy, University of Rajshahi
Dr. Md. Saiful Islam, Associate Professor, Department of Laboratory Medicine, Bangabandhu Sheikh Mujib Medical University, Dhaka ,Bangladesh

APPENDIX C

ETHICAL CLEARANCE CERTIFICATE

UNIVERSITY OF RAJSHAHI
INSTITUTE OF BIOLOGICAL SCIENCES
Rajshahi 6205, Bangladesh
Tel: +880-721- 750928, Cell. +880-01716288356, Fax + 880-721-711127, +880-721-750064
E-mail: director_ibsc@ru.ac.bd/ monzurhb@gmail.com; http: // www.dept.ru.ac.bd/ibsc

Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC)
for Experimentations on Animal, Human, Microbes and Living Natural Sources

(Approved in the Resolution No. of the 71th meeting of the Board of Governors of the Institute of Biological Sciences and Resolution No. 57 of the meeting of the Syndicate of the University of Rajshahi)

Memo No: 82/320/IAMEBBC/IBSC 20 August, 2017

Certificate

This is to certify that the project title "CORRELATION BETWEEN HEAVY METALS AND NEONATAL HYPERBILIRUBINEMIA IN DHAKA" Submitted by Mohammad Anwar Hossain, M.Phil Fellow, Institute Biological Sciences, University of Rajshahi has been approved by the IAMEBBC in its resolution no. 02 of the 15th meeting held on 20th August, 2017.

Name of Chairman: Prof. Dr. M. Monzur Hossain

Monzur Hossain
29/11/17
Signature With Date
Director
Institute of Biological Sciences
Rajshahi University

APPENDIX D

CONSENT FORM

Title of the study:

TITLE: CORRELATION BETWEEN HEAVY METALS AND NEONATAL HYPERBILIRUBINEMIA AMONG THE PATIENTS ATTENDED AT BANGABANDHU SHEIKH MUJIB MEDICAL UNIVERSITY

Principal investigator: Mohammad Anwar Hossain

অবহিতক্রমে সম্মতিপত্র

গবেষণার নামঃ নবজাতকের রক্তের জন্ডিস ও ভারী ধাতব পদার্থের মধ্যে পারস্পরিক সম্পর্ক নিরূপণ
প্রধান গবেষকঃ মুহাম্মদ আনোয়ার হোসেন ।

এই সম্মতিপত্রের উদ্দেশ্য হলো আপনাকে প্রয়োজনীয় তথ্য প্রদান করা, যে তথ্যগুলো আপনাকে সিদ্ধান্ত নিতে সাহায্য করবে, আপনি এই গবেষণায় অংশগ্রহণ করবেন কিনা?

উদ্দেশ্য : এই বিস্তারিত প্রশ্নমালার মাধ্যমে নবজাতক ও নবজাতকের মাতা সম্পর্কে বিস্তারিত তথ্যাবলী সংগ্রহ করা হবে। জন্মের পরে নাবজাতকের নাড়ী এবং শিরা থেকে রক্ত সংগ্রহ করে পরীক্ষার জন্য ক্লিনিক্যাল প্যাথলজি বিভাগের পরীক্ষাগারে পাঠানো হবে। পরীক্ষাগারে নবজাতকের রক্তের সিরাম ম্যাগনেশিয়াম,জিংক এবং কপার এর পরিমান নিরূপন করা হবে। এই গবেষাণার পরীক্ষাগুলো বঙ্গবন্ধু শেখ মুজিব মেডিকেল বিশ্ববিদ্যালয়, ক্লিনিক্যাল প্যাথলজি বিভাগে করা হবে। আপনি যদি এই গবেষণায় অংশগ্রহণ করতে সম্মত থাকেন তাহলে গবেষণায় নিয়োজিত চিকিৎসক সে সম্পর্কিত কিছু প্রশ্ন আপনাকে করবেন ।

গবেষণায় অংশগ্রহণের সুবিধাদিঃ এই গবেষণার মাধ্যমে প্রাপ্ত তথ্য এবং গবেষণালব্দ জ্ঞান এই নবজাতক চিকিৎসার মাধ্যমে উপকৃত হবে এবং ভবিষ্যতে শিশুদের চিকিৎসার ক্ষেত্রে গুরুত্বপূর্ণ ভূমিকা রাখতে পারে। অভিভাবক প্রয়োজন অনুভব করলে যে কোন সময় এই গবেষণা থেকে নাম প্রত্যাহার করতে পারবেন। বিনামূল্যে অতি দ্রুত ও সঠিক নির্ণয়ের মাধ্যমে আপানার/ রোগীর সময়মত নির্দিষ্ট চিকিৎসা গ্রহণের সুযোগ পাবেন । এই গবেষণা বাংলাদেশে চিকিৎসকদের এই রোগ সম্পর্কে আরো জানতে সহায়তা করবে।

গবেষণার ঝুঁকিঃ এই গবেষণায় কোন ঝুকি নেই । রোগীর নূন্যতম ঝুকি কিভাবে কমানো যায় সেদিকে লক্ষ্য রাখা হবে।

খরচঃ এই গবেষণায় অংশগ্রহণের জন্য আপনার কোন খরচ নাই বা আপনাকে কোন টাকা পয়সা দেয়া হবে না ।

গোপনীয়তা: গবেষণা চলাকারীন ও পরবর্তীতে সকর তথ্য কঠোরভাবে গোপন রাখা হবে এবং এই সকল ব্যাপারে এমন কোথাও আলাপ আলোচনা করা হবে না যাতে নবজাতক এবং নবজাতকের পিতা মাতা বিব্রতবোধ করতে পারে।

প্রশ্নাবলী: যদি আপনার কোন প্রশ্ন থাকে তবে দয়া করে জিজ্ঞাসা করবেন । আমরা তার উত্তর প্রদান করার যথাসাধ্য চেষ্টা করবো। যদি ভবিষ্যতে আপানার অতিরিক্ত কোন প্রশ্ন থাকে তাহলে গকেষণারত ডাক্তারের সাথে যোগাযোগ করতে পারেন।

সম্মতির স্বীকারোক্তি: আমি গবেষনায় নিয়োজিত চিকিৎসক-এর সাথে (যিনি আমার/ রোগীর শারীরিক পরীক্ষা করবেন) এই গবেষণা নিয়ে আলোচনায় সন্তুষ্টি প্রকাশ করছি। আমি এটা বুঝেছি যে গবেষণায় অংশগ্রহণ স্বেচ্ছামূলক এবং আমি যে কোন সময় কোন বাধ্যবধকতা ছাড়াই গবেষণা থেকে আমাকে/ নবজাতককে বিরত রাখতে পারি। আমি উপরোক্ত শর্তগুলো পড়েছি/ আমার সম্মখে পঠিত হয়েছেএবং স্বেচ্ছায় গবেষণায় অংশগ্রহণ করতে সম্মতি জ্ঞাপন করছি।

গবেষকের স্বাক্ষর

তারিখ:

নবজাতকের অভিভাবকের স্বাক্ষর/ স্বাক্ষীর স্বাক্ষর/ বৃদ্ধাঙ্গুলির ছাপ

তারিখ:

APPENDIX E

CASE RECORD PROFORMA

(Enter a √ in the appropriate box)

TITLE:CORRELATION BETWEEN HEAVY METALS AND NEONATAL HYPERBILIRUBINEMIA AMONG THE PATIENTS ATTENDED AT BANGABANDHU SHEIKH MUJIB MEDICAL UNIVERSITY

Principal investigator**:** Mohammad Anwar Hossain

1. Centre Name: Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka

2. **OPD No:** ☐ **3. Registration No:** ☐ 4. **Date:** ☐

5. Name of the Subject:

6. Subject Sl. No.: ☐ **7. Group No:** ☐ CASE/CONTROL

8. Date of Birth: Days……. Months……Year…….

9. Sex: Male or Female

10. Body weight of Neonate:

11. Address:

_____

_____

12. Telephone/contact No(Fathers/Guardian):

_____

13. Mothers Occupation: Housewife/Service Holder

14. History of ANC Checkup: Regular/Irregular/No

15. History of Past Illness: No/Yes (DM, HTN, IHD, Others)

16. Mothers Socioeconomic Condition: Upper/Upper Middle/Lower Middle/Lower

17. Inclusion criteria:

- Neonates with both sex and age up to 28 days.
- The neonates of hyperbilirubinemia according to their specified age group were included as cases.
- The healthy neonates who had normal level of bilirubin according to their specified age group were included as controls.
- The level of serum bilirubin >140 mg/L(>14mg/dl) in hyperbilirubinemia and <140 mg/L(<14mg/dl) in healthy neonates according to their specified age group.

18. Exclusion criteria:

- Age more than 28 days.
- Current history of illness with severe renal, respiratory and cardiovascular diseases etc.

19. Laboratory Investigations:

- Serum total bilirubin level in blood----------mg/dl
- Serum magnesium (Mg) level in blood----------mg/dl
- Serum zinc (Zn) level in blood----------µg/dl
- Serum copper (Cu) level in blood---------- µg/dl

_____

Signature of Investigator

কেইস রেকর্ড প্রোফরমা (In Bangla)

(Enter a √ in the appropriate box)

শিরোনাম : CORRELATION BETWEEN HEAVY METALS AND NEONATAL HYPERBILIRUBINEMIA AMONG THE PATIENTS ATTENDED AT BANGABANDHU SHEIKH MUJIB MEDICAL UNIVERSITY

১. কেন্দের নাম: বঙ্গবন্ধু শেখ মুজিব মেডিক্যাল বিশ্ববিদ্যালয় **শাহবাগ, ঢাকা, বাংলাদেশ**

২. **বহিঃ বিভাগ:** ☐ ৩. **রেজি.নং :** ☐ ৪. **তারিখ** ☐

৫. রোগির নাম : _____

৬. রোগির সিরিয়াল নং .: ☐ **৭. গ্রুপ নং:** CASE/CONTROL

৮. জন্ম তারিখ :দিন……. মাস……বৎসর…….

৯. লিঙ্গ:পুরুষ /মহিলা

১০. নবজাতকের ওজন

১১. ঠিকানা : _____গ্রাম :_____পোষ্ট :_____ থানা :_____

জেলা :_____

১২ . টেলিফোন নং /contact No(পিতা/অভিভাবক): _____

১৩. মাতার পেশা : গৃহিনী/চাকুরিজিবী

১৪. ইতিহাস অফ এন্টিনেটাল চেকআপ:নিয়ামিত/অনিয়ামিত/ না

১৫. অতীত অসুস্থ্যতার ইতিহাস: না/হ্যাঁ (DM, HTN, IHD, Others)

১৬.আর্থ সামাজিক অবস্থা : Upper/Upper Middle/Lower Middle/Lower

১৭. Inclusion criteria:

- Neonates with both sex and age up to 28 days.
- The neonates of hyperbilirubinemia according to their specified age group were included as cases.
- The healthy neonates who had normal level of bilirubin according to their specified age group were included as controls.
- The level of serum bilirubin >140 mg/L(>14mg/dl) in hyperbilirubinemia and <140 mg/L(<14mg/dl) in healthy neonates according to their specified age group.

১৮. Exclusion criteria:

- Age more than 28 days.
- Current history of illness with severe renal, respiratory and cardiovascular diseases etc.

১৯ . ল্যাবরেটরি পরিক্ষা:

- রক্তের সিরাম বিলিরুবিন ---------mg/dl
- রক্তের সিরাম ম্যাগনেসিয়াম ---- mg/dl
- রক্তের সিরাম জিংক---------- µg/dl
- রক্তের সিরাম কপার ---------- µg/dl

_____

Signature of Investigator

APPENDIX F

Biochemistry report format

বঙ্গবন্ধু শেখ মুজিব মেডিক্যাল বিশ্ববিদ্যালয়

Bangabandhu Sheikh Mujib Medical University

DEPARTMENT OF LABORATORY MEDICINE

Biochemistry Report Format

ID No- 2243/222	Receving date:3/4/18	Delivery date: 5/4/18	
Name: B/O Farzana		Age:2 Days	Female
Ward: opd	BED:		
Refd. By:			
Sample	Blood (Serum)		
Test Name	Serum Bilirubin, Serum Magnesium, Serum Zinc, Serum Copper		

Test are done by Siemens Automated Biochemistry Analyzer Dimension *RxL*, MAX, USA & Semi automated Biochemistry Analyzer, Evolution-3000, Italy.

Name of the Test	Result	Unit	Reference Range
Serum Bilirubin (Total)	16.5	mg/dl	Adult 0.2- 1.0 mg/dl New born upto 14.0 mg/dl (Upto 24 hours 1.0-8.0 mg/dl Upto 48 hours 6.0-12.0 mg/dl 3 to 5 days 10.0 -14.0 mg/dl)
Serum Magnesium	2.5	mg/dl	Newborn, 2 to 4 days 1.5 to 2.2 mg/dl 5 months to 6 years 1.7 to 2.3 mg/dl 6 to 12 years 1.7 to 2.1 mg/dl 12 to 20 years 1.7 to 2.2 mg/dl Adult 1.6 to 2.6 mg/dl
Serum Zinc	47.1	µg/dl	Adult Male- 72.6-127.0 µg/dl Adult Female- 70.0-114.0 µg/dl Neonate- 49.5-99.7 µg/dl
Serum Copper	66.8	µg/dl	Adult Male- 80.0-140.0 µg/dl Adult Female- 80.0-155.0 µg/dl Neonate- 12.0-67.0 µg/dl Child. up to 10 years-.30.0-150.0 µg/dl

Done by
Mohammad Anwar Hossain

Consultant
Dr. Md. Saiful Islam
MBBS.M.Phil (Cl. Path)
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APPENDIX G

Master Chart

Control Group														Study Group							
No. of Patient	**Age (Days)**	**Sex**	**Body weight**	**Mothers Occupation**	**H/o ANC**	**Socioeconomic History**	**S. Billirubin**	**S. Mg**	**S. Zinc**	**S. Copper**	**No. of Patient**	**Age (Days)**	**Sex**	**Body weight**	**Mothers Occupation**	**H/o ANC**	**Socioeconomic History**	**S. Billirubin**	**S. Mg**	**S. Zinc**	**S. Copper**
1	1	Male	2.6	H. Wife	Regular	UM	34	19	0.85	0.605	1	4	Female	1.80	H. Wife	Regular	UM	194	25	0.523	0.669
2	1	Male	2.7	H. Wife	Regular	UM	52	22	0.68	0.382	2	1	Male	2.6	H. Wife	Regular	UM	165	27	0.477	0.662
3	5	Male	2.9	H. Wife	Regular	UM	46	19	0.556	0.401	3	2	Male	1.9	H. Wife	Regular	LM	172	25	0.471	0.669
4	1	Female	2.5	H. Wife	Regular	UM	37	24	0.627	0.516	4	1	Male	2.5	H. Wife	Regular	LM	157	19	0.458	0.694
5	6	Male	4.0	Service	Regular	UP	50	20	0.556	0.567	5	4	Female	4.5	Service	Regular	UM	205	25	0.523	0.662
6	1	Male	2.6	H. Wife	Regular	LM	36	22	0.627	0.586	6	4	Male	2.6	H. Wife	Regular	UM	203	27	0.477	0.701
7	6	Male	2.8	H. Wife	Regular	UM	33	20	0.815	0.561	7	2	Male	2.4	H. Wife	Regular	UM	190	24	0.484	0.694
8	1	Male	2.7	H. Wife	Regular	LM	48	22	0.719	0.395	8	1	Male	2.7	H. Wife	Regular	UM	172	27	0.451	0.803
9	6	Male	2.5	Service	Regular	UM	50	19	0.513	0.61	9	2	Female	2.5	Service	Regular	UM	171	28	0.458	0.771
10	1	Female	2.7	H. Wife	Irregular	LM	29	19	0.641	0.242	10	2	Male	2.3	H. Wife	Regular	LM	181	27	0.51	0.822
11	24	Female	2.6	H. Wife	Regular	UM	30	18	0.961	0.618	11	2	Female	2.4	H. Wife	Regular	UP	190	23	0.516	0.669
12	1	Male	2.5	H. Wife	Regular	UM	40	19	0.693	0.605	12	1	Male	2.5	H. Wife	Regular	LO	166	24	0.542	0.847
13	5	Female	3.2	H. Wife	Regular	UP	51	17	0.529	0.369	13	1	Male	2.2	H. Wife	Regular	LO	148	20	0.51	0.771
14	5	Female	2.7	H. Wife	Regular	LO	46	18	0.542	0.357	14	2	Female	2.7	H. Wife	Irregular	LM	166	26	0.51	0.809
15	7	Female	2.7	H. Wife	Regular	LM	30	19	0.667	0.223	15	3	Male	2.6	H. Wife	Regular	UM	182	20	0.477	0.79
16	1	Female	2.5	H. Wife	Regular	LM	53	20	0.549	0.278	16	3	Male	2.5	H. Wife	Regular	UM	156	22	0.641	0.898
17	22	Female	2.8	H. Wife	Regular	UP	38	19	0.621	0.338	17	3	Female	2.8	H. Wife	Regular	UM	204	20	0.523	0.809
18	1	Male	2.9	Service	Regular	UM	48	20	0.712	0.503	18	4	Male	2.4	Service	Regular	UM	190	24	0.484	0.688
19	1	Male	2.5	H. Wife	Irregular	UM	40	18	0.719	0.637	19	2	Male	2.5	H. Wife	Regular	UM	171	25	0.569	0.822
20	4	Male	3.2	H. Wife	Regular	UM	55	19	0.817	0.331	20	2	Male	2.2	H. Wife	Regular	UM	174	27	0.49	0.86
21	1	Male	2.7	H. Wife	Regular	UM	33	20	0.588	0.28	21	5	Male	2.7	Service	Regular	UM	201	26	0.523	0.809
22	1	Male	2.8	H. Wife	Regular	UM	34	19	0.641	0.369	22	3	Male	2.8	H. Wife	Regular	UM	189	24	0.458	0.675
23	1	Male	2.6	H. Wife	Regular	UM	30	19	0.549	0.433	23	3	Male	2.6	H. Wife	Regular	UM	183	28	0.477	0.885
24	8	Male	2.8	H. Wife	Regular	UM	36	20	0.928	0.439	24	1	Female	2.8	Service	Regular	LM	148	**24**	0.516	0.873
25	1	Female	2.6	H. Wife	Regular	UM	34	19	0.765	0.357	25	3	Male	2.6	H. Wife	Regular	LM	182	24	0.497	0.898
26	13	Male	2.8	H. Wife	Irregular	LO	28	20	0.797	0.478	26	3	Male	2.8	H. Wife	Regular	LM	154	25	0.49	0.924

27	1	Female	2.7	H. Wife	Regular	LO	35	18	0.523	0.242	27	1	Male	2.7	H. Wife	Regular	UM	142	24	0.477	0.949
28	22	Male	2.8	H. Wife	Regular	LM	34	24	0.804	0.535	28	1	Male	2.8	H. Wife	Regular	UM	159	25	0.456	0.828
29	1	Male	2.9	Service	Regular	UM	32	18	0.778	0.605	29	1	Female	3	Service	Regular	UM	147	26	0.471	0.803
30	9	Male	2.9	H. Wife	Regular	UM	50	20	0.915	0.414	30	1	Male	2.9	H. Wife	Regular	UM	160	19	0.497	0.809
31	1	Female	2,8	H. Wife	Regular	UM	51	18	0.699	0.656	31	2	Female	3	H. Wife	Regular	LM	150	19	0.484	0.732
32	1	Female	2.9	H. Wife	Regular	LO	28	19	0.802	0.299	32	2	Male	2.9	H. Wife	Irregular	LM	197	25	0.516	0.809
33	1	Male	2.8	H. Wife	Regular	UM	29	17	0.68	0.58	33	3	Male	2.8	H. Wife	Regular	UM	180	19	0.484	0.904
34	10	Male	2.7	H. Wife	Regular	UM	41	19	0.627	0.58	34	2	Male	2.7	H. Wife	Regular	UP	168	23	0.497	0.847
35	1	Male	2.6	H. Wife	Regular	UM	50	19	0.699	0.452	35	1	Male	2.6	H. Wife	Regular	UM	145	24	0.451	0.739
36	1	Female	3.2	H. Wife	Regular	UM	30	19	0.876	0.393	36	1	Male	3.2	H. Wife	Irregular	UM	171	25	0.523	0.777
37	1	Female	3	Service	Regular	UM	25	20	0.549	0.362	37	1	Male	3	H. Wife	Regular	UM	143	24	0.51	0.949
38	1	Female	2.7	H. Wife	Regular	LM	27	18	0.686	0.293	38	1	Female	2.7	H. Wife	Regular	UM	142	27	0.516	0.771
39	2	Female	3.5	H. Wife	Regular	LM	53	20	0.601	0.478	39	2	Male	3.5	Service	Regular	UM	154	23	0.497	0.669
40	2	Male	2.8	H. Wife	Regular	UM	50	23	0.582	0.299	40	2	Female	2.8	H. Wife	Regular	UM	158	22	0.516	0.669
41	2	Male	2.6	H. Wife	Regular	LO	51	22	0.758	0.497	41	2	Female	2.6	H. Wife	Regular	UP	154	27	0.451	0.904
42	1	Female	2.6	H. Wife	Regular	UM	51	20	0.895	0.605	42	1	Male	2.4	H. Wife	Regular	UM	170	25	0.451	0.873
43	3	Female	3.1	H. Wife	Regular	UM	36	22	0.706	0.595	43	2	Male	1.7	H. Wife	Regular	UM	170	25	0.549	0.669
44	3	Male	2.7	Service	Regular	LM	38	19	0.673	0.59	44	3	Female	2.7	H. Wife	Regular	UM	185	23	0.503	0.701
45	4	Male	2.9	H. Wife	Irregular	UM	44	18	0.601	0.224	45	2	Female	2.1	H. Wife	Regular	LM	172	25	0.51	0.809
46	2	Male	3,1	H. Wife	Regular	UM	53	20	0.667	0.274	46	3	Female	3	H. Wife	Regular	LM	185	27	0.523	0.739
47	5	Male	3.1	H. Wife	Regular	LM	35	19	0.601	0.248	47	8	Female	3.1	Service	Regular	UM	208	26	0.464	0.682
48	3	Male	2.8	H. Wife	Regular	UM	39	21	0.542	0.382	48	3	Female	1.8	H. Wife	Regular	UM	179	25	0.477	0.892
49	1	Male	3.2	H. Wife	Regular	UM	51	19	0.529	0.185	49	1	Male	3.2	H. Wife	Irregular	LM	157	23	0.523	0.771
50	1	Female	2.6	H. Wife	Regular	UM	28	21	0.595	0.236	50	1	Male	2.6	H. Wife	Regular	LM	145	24	0.49	0.72
51	4	Male	3.2	H. Wife	Regular	LM	36	22	0.536	0.299	51	3	Male	2.2	H. Wife	Regular	LM	195	28	0.497	0.72
52	1	Female	2.6	H. Wife	Regular	UM	36	22	0.627	0.312	52	1	Female	2.6	H. Wife	Regular	UM	145	22	0.503	0.701
53	2	Female	2.9	H. Wife	Regular	LM	28	26	0.673	0.382	53	2	Female	2.9	Service	Regular	UP	209	26	0.529	0.803
54	5	Female	3.3	Service	Regular	LO	29	19	0.856	0.624	54	1	Male	2.0	H. Wife	Regular	UM	171	24	0.497	0.822
55	7	Male	2.8	H. Wife	Regular	UP	32	18	0.778	0.529	55	5	Male	2.8	H. Wife	Regular	UM	156	22	0.51	0.904
56	10	Female	2.5	Service	Regular	LM	29	19	0.745	0.274	56	8	Female	2.5	H. Wife	Regular	LM	170	19	0.497	0.675
57	2	Male	2.7	H. Wife	Regular	LM	53	21	0.902	0.471	57	9	Male	2.7	H. Wife	Regular	UM	166	25	0.438	0.796
58	11	Female	2.9	H. Wife	Irregular	UM	32	21	0.765	0.541	58	2	Female	2.9	H. Wife	Regular	LM	165	26	0.503	0.707
59	1	Female	2.6	H. Wife	Regular	LM	30	18	0.68	0.376	59	3	Female	2.6	Service	Regular	UP	192	25	0.51	0.904
60	1	Female	2.8	H. Wife	Regular	UM	36	19	0.66	0.605	60	4	Male	2.8	H. Wife	Regular	UM	164	24	0.497	0.637
61	13	Female	3.1	H. Wife	Regular	UM	32	21	0.51	0.598	61	1	Male	3.1	H. Wife	Regular	UM	140	21	0.49	0.682
62	2	Male	2.7	H. Wife	Regular	UM	53	19	0.797	0.338	62	2	Male	2.7	H. Wife	Irregular	UM	167	28	0.477	0.71
63	6	Female	2.9	Service	Regular	UM	46	20	0.66	0.439	63	3	Male	2.9	H. Wife	Regular	UM	188	27	0.484	0.662
64	7	Female	2.9	H. Wife	Regular	UM	36	22	0.549	0.223	64	2	Female	3.o	H. Wife	Regular	LM	180	22	0.49	0.65

65	8	Male	2.7	H. Wife	Regular	LM	35	20	0.601	0.369	65	2	Male	1.8	Service	Regular	UM	154	19	0.477	0.777
66	5	Male	3.1	H. Wife	Regular	LM	43	19	0.579	0.65	66	1	Male	3.1	H. Wife	Regular	UM	171	28	0.523	0.936
67	1	Male	2.9	H. Wife	Regular	UM	36	21	0.797	0.529	67	3	Male	2.9	H. Wife	Regular	LM	182	25	0.529	0.809
68	14	Male	3	H. Wife	Regular	UM	30	22	0.66	0.28	68	1	Male	2.8	Service	Regular	LM	141	25	0.542	0.669
69	4	Male	3.1	Service	Regular	UM	50	20	0.863	0.522	69	2	Male	3.1	H. Wife	Regular	UM	150	20	0.497	0.713
70	2	Female	2.8	H. Wife	Regular	LM	49	19	0.771	0.242	70	2	Male	1.8	Service	Regular	LM	140	19	0.464	0.898
71	5	Male	3.2	H. Wife	Regular	LM	51	18	0.856	0.548	71	2	Male	3.2	H. Wife	Regular	UM	165	22	0.608	0.739
72	3	Female	2.6	H. Wife	Irregular	LO	38	19	0.595	0.427	72	2	Male	2.6	H. Wife	Regular	UM	167	23	0.536	0.809
73	1	Male	2.9	H. Wife	Regular	UM	28	17	0.68	0.312	73	3	Male	2.2	H. Wife	Regular	UM	190	22	0.562	0.879
74	1	Female	2.6	H .Wife	Regular	UM	51	20	0.608	0.325	74	1	Male	2.6	H. Wife	Regular	UM	149	22	0.458	0.873
75	4	Male	2.9	H. Wife	Regular	UM	42	17	0.549	0.306	75	2	Male	2.9	H. Wife	Regular	LM	162	26	0.503	0.904
76	1	Female	2.5	H. Wife	Regular	UP	52	19	0.85	0.586	76	1	Female	2	H. Wife	Regular	UM	145	23	0.438	0.809
77	2	Female	2.8	H. Wife	Regular	UM	29	21	0.706	0.23	77	2	Male	2.8	H. Wife	Regular	UM	154	21	0.471	0.682
78	5	Female	2.5	H. Wife	Regular	UM	27	20	0.621	0.248	78	2	Male	2.5	Service	Regular	UP	174	24	0.516	0.764
79	7	Male	2.7	Service	Regular	UM	27	21	0.693	0.293	79	2	Male	2.7	H. Wife	Regular	UM	175	26	0.523	0.911
80	10	Male	2.9	H. Wife	Regular	LM	26	18	0.523	0.344	80	10	Male	2.9	H. Wife	Regular	UM	205	24	0.497	0.726
81	2	Male	2.6	H. Wife	Irregular	LM	30	20	0.791	0.35	81	2	Female	2.6	H. Wife	Regular	UM	172	20	0.503	0.688
82	11	Female	2.8	H. Wife	Regular	LO	52	20	0.542	0.245	82	1	Female	2.8	Service	Regular	UM	140	18	0.451	0.809
83	1	Male	3.1	H. Wife	Regular	LM	40	18	0.68	0.242	83	1	Male	3.1	H. Wife	Regular	UM	140	24	0.497	0.752
84	1	Male	2.8	H. Wife	Regular	LM	36	20	0.752	0.439	84	1	Female	1.8	H. Wife	Irregular	UM	160	22	0.451	0.669
85	13	Male	2.6	H. Wife	Regular	UM	36	23	0.693	0.306	85	1	Male	2.6	Service	Regular	LM	153	23	0.569	0.764
86	2	Male	2.9	H. Wife	Regular	UM	45	22	0.654	0.24	86	3	Female	1.9	H. Wife	Regular	LO	200	24	0.503	0.688
87	2	Female	2.5	Service	Regular	UM	43	22	0.601	0.217	87	1	Male	2.5	H. Wife	Regular	UM	150	19	0.458	0.79
88	7	Male	3.9	H. Wife	Regular	UP	49	19	0.621	0.503	88	2	Male	4.1	H. Wife	Regular	LM	160	24	0.451	0.688
89	8	Male	2.6	H. Wife	Regular	UM	45	20	0.608	0.21	89	1	Female	2.6	H. Wife	Regular	UM	172	28	0.51	0.841
90	5	Female	2.7	H. Wife	Regular	LM	43	21	0.81	0.223	90	2	Male	2.4	Service	Regular	UM	175	25	0.503	0.701
91	1	Female	2.7	H. Wife	Regular	LM	36	18	0.699	0.363	91	1	Male	2.7	H. Wife	Regular	LM	181	22	0.51	0.662
92	14	Female	2.5	H. Wife	Regular	LM	42	22	0.589	0.39	92	1	Female	2.5	H. Wife	Regular	UM	182	25	0.523	0.898
93	2	Male	3.3	H. Wife	Regular	LO	38	22	0.882	0.44	93	3	Male	2.3	H. Wife	Regular	UP	197	23	0.503	0.682
94	1	Male	2.9	H. Wife	Regular	LO	34	19	0.696	0.388	94	2	Female	2.4	H. Wife	Regular	UM	157	22	0.471	0.809
95	3	Male	2.5	Service	Regular	UM	31	20	0.697	0.479	95	1	Female	2.5	Service	Regular	UM	158	21	0.51	0.688
96	1	Female	3.2	H. Wife	Regular	UM	35	21	0.623	0.342	96	8	Female	2.2	H. Wife	Regular	LO	140	24	0.438	0.701
97	2	Male	2.7	H. Wife	Regular	UM	36	23	0.704	0.545	97	1	Male	2.1	H. Wife	Regular	UM	142	23	0.49	0.662
98	1	Male	2.7	H. Wife	Regular	UM	33	19	0.678	0.609	98	4	Male	2.7	H. Wife	Regular	UM	198	26	0.529	0.669
99	3	Male	2.5	H. Wife	Regular	UM	49	20	0.815	0.514	99	8	Female	2.5	H. Wife	Irregular	UM	140	22	0.549	0.771
100	2	Female	3.3	H. Wife	Regular	UM	51	19	0.68	0.298	100	1	Female	2.3	H. Wife	Regular	UM	156	25	0.529	0.809
101	2	Male	2.7	H. Wife	Regular	UM	39	20	0.699	0.266	101	1	Male	2.4	Service	Regular	UP	143	22	0.536	0.809
102	3	Female	2.5	H. Wife	Irregular	UM	40	21	0.551	0.388	102	1	Male	2.5	H. Wife	Regular	UM	148	20	0.49	0.675

103	1	Male	3.2	H. Wife	Regular	UP	48	20	0.531	0.255	103	1	Female	2.2	H. Wife	Regular	UM	141	23	0.49	0.662
104	1	Female	2.7	H. Wife	Regular	LM	31	18	0.585	0.246	104	3	Male	2.7	H. Wife	Regular	UM	188	24	0.477	0.854
105	2	Male	2.8	Service	Regular	UM	39	21	0.601	0.309	105	4	Female	2.8	Service	Regular	UM	200	26	0.529	0.79
106	1	Female	2.6	H. Wife	Regular	UM	37	19	0.629	0.316	106	2	Female	2.5	H. Wife	Regular	UM	174	25	0.503	0.828
107	2	Male	2.8	H. Wife	Regular	UM	30	23	0.662	0.39	107	1	Female	2.3	H. Wife	Regular	LM	167	25	0.549	0.841
108	3	Male	2.6	Service	Regular	UM	29	19	0.809	0.634	108	2	Male	2.6	H. Wife	Regular	UM	170	26	0.497	0.822
109	4	Male	2.8	H. Wife	Irregular	LM	33	18	0.77	0.502	109	8	Male	2.8	H. Wife	Regular	UM	162	22	0.49	0.771
110	1	Male	2.7	H. Wife	Regular	LO	28	20	0.741	0.289	110	1	Female	2.7	H. Wife	Regular	UM	146	19	0.497	0.682
111	2	Male	2.8	H. Wife	Regular	LM	50	21	0.881	0.462	111	2	Female	2.8	Service	Regular	UM	190	25	0.49	0.758
112	1	Female	2.6	H. Wife	Regular	LM	34	19	0.752	0.526	112	1	Male	3.5	H. Wife	Regular	UM	159	24	0.516	0.682
113	2	Male	2.9	H. Wife	Regular	UM	38	20	0.602	0.269	113	2	Male	2.9	H. Wife	Regular	UM	180	21	0.497	0.809
114	3	Male	2.7	Service	Regular	UM	41	19	0.558	0.382	114	1	Female	2.6	H. Wife	Regular	UM	140	20	0.471	0.752
115	1	Male	2.9	H. Wife	Regular	UM	42	21	0.539	0.235	115	10	Male	2.9	Service	Regular	UM	156	23	0.503	0.764
116	1	Female	2.8	H. Wife	Regular	UM	32	19	0.599	0.251	116	2	Male	2.8	H. Wife	Regular	UM	164	22	0.503	0.688
117	2	Male	2.7	H. Wife	Regular	UM	37	21	0.623	0.298	117	3	Female	2.7	Service	Regular	LM	190	27	0.49	0.675
118	1	Female	2.6	H. Wife	Regular	LM	39	20	0.635	0.305	118	2	Male	2.6	H. Wife	Regular	LM	200	26	0.523	0.701
119	2	Male	3.2	H. Wife	Regular	LM	47	22	0.576	0.392	119	3	Female	3.2	H. Wife	Regular	LM	169	24	0.458	0.79
120	3	Male	3.3	H. Wife	Regular	UP	31	19	0.745	0.592	120	14	Male	3.3	H. Wife	Regular	LM	195	27	0.497	0.72
121	2	Male	2.7	H. Wife	Regular	UM	36	18	0.687	0.481	121	1	Male	1.7	H. Wife	Regular	LM	160	21	0.51	0.656
122	1	Male	3.5	Service	Regular	UM	44	20	0.706	0.299	122	1	Male	3.5	Service	Regular	LO	157	24	0.444	0.637
123	2	Male	2.8	H. Wife	Regular	UM	51	21	0.72	0.456	123	1	Female	2.8	H. Wife	Regular	UM	149	23	0.614	0.949
124	1	Female	2.6	H. Wife	Regular	UM	36	19	0.738	0.512	124	3	Male	2.6	H. Wife	Regular	UM	192	24	0.529	0.898
125	2	Male	2.8	H. Wife	Regular	UM	40	20	0.598	0.278	125	2	Male	2.4	H. Wife	Regular	UM	180	25	0.49	0.93
126	3	Male	2.9	H. Wife	Regular	UM	41	22	0.559	0.395	126	2	Male	1.6	H. Wife	Regular	LM	174	26	0.51	0.732
127	1	Male	2.7	H. Wife	Regular	UM	42	20	0.561	0.235	127	4	Male	2.7	H. Wife	Regular	UM	201	26	0.562	0.815
128	1	Female	2.7	H. Wife	Irregular	LM	33	19	0.606	0.252	128	1	Male	2.1	H. Wife	Regular	UM	163	29	0.458	0.809
129	2	Male	3	H. Wife	Regular	LM	37	20	0.579	0.315	129	2	Male	2.8	Service	Regular	UM	171	26	0.471	0.917
130	1	Female	3.1	H. Wife	Regular	LO	40	21	0.618	0.324	130	1	Female	3.1	H. Wife	Regular	UM	146	20	0.477	0.803
131	2	Male	2.8	H. Wife	Regular	LM	38	18	0.66	0.397	131	4	Female	1.8	H. Wife	Regular	UM	208	25	0.562	0.669
132	3	Male	3.2	Service	Regular	UM	28	19	0.781	0.601	132	9	Female	3.2	H. Wife	Regular	UP	174	26	0.49	0.675
133	2	Male	2.6	H. Wife	Regular	UM	39	18	0.748	0.495	133	2	Female	2.6	H. Wife	Irregular	UM	175	21	0.477	0.662
134	1	Male	3.2	H. Wife	Regular	LM	36	20	0.718	0.296	134	1	Female	2.2	H. Wife	Regular	UM	152	22	0.497	0.643
135	2	Male	2.6	H. Wife	Regular	UM	48	21	0.81	0.471	135	2	Male	2.4	H. Wife	Regular	UM	163	25	0.51	0.86
136	1	Female	2.9	H. Wife	Regular	UM	35	19	0.727	0.513	136	2	Male	2.9	H. Wife	Irregular	UM	161	25	0.595	0.771
137	2	Male	3,2	Service	Regular	UM	40	18	0.652	0.391	137	3	Male	2.2	Service	Regular	LM	190	24	0.512	0.669
138	3	Male	2.8	H. Wife	Regular	UM	38	20	0.752	0.543	138	3	Female	2.8	H. Wife	Regular	UM	200	20	0.536	0.745
139	2	Male	2.5	H. Wife	Regular	UM	39	18	0.708	0.482	139	15	Female	2.5	H. Wife	Regular	LM	157	23	0.556	0.904
140	1	Male	2.7	H. Wife	Regular	UM	42	22	0.628	0.329	140	8	Male	2.7	H. Wife	Regular	LM	166	25	0.516	0.879

141	4	Male	2.9	H. Wife	Regular	LM	39	19	0.665	0.497	141	4	Female	2.9	H. Wife	Regular	UM	202	21	0.542	0.809
142	2	Male	2.6	H. Wife	Regular	LM	38	20	0.691	0.55	142	1	Male	2.6	H. Wife	Regular	UM	156	24	0.601	0.707
143	1	Male	2.8	H. Wife	Regular	LM	40	19	0.708	0.498	143	2	Male	2.8	H. Wife	Regular	UM	155	21	0.477	0.732
144	1	Male	3.1	H. Wife	Regular	LO	42	21	0.689	0.396	144	3	Male	3.1	Service	Regular	UM	190	25	0.51	0.828
145	1	Male	2.7	H. Wife	Regular	LO	46	20	0.71	0.461	145	1	Female	2.7	H. Wife	Regular	UP	171	24	0.529	0.688
146	2	Female	2.9	H. Wife	Regular	LM	37	19	0.705	0.512	146	1	Female	2.9	Service	Regular	UM	143	19	0.497	0.809
147	1	Male	3.1	H. Wife	Irregular	UM	41	22	0.682	0.39	147	7	Female	3.1	H. Wife	Regular	UM	199	23	0.529	0.732
148	1	Male	2.9	Service	Regular	UM	39	21	0.703	0.533	148	10	Female	1.8	H. Wife	Regular	LM	142	24	0.588	0.662
149	3	Male	3.1	H. Wife	Regular	UM	40	19	0.698	0.481	149	10	Female	3.1	H. Wife	Regular	UP	140	22	0.608	0.65
150	1	Male	2.9	H. Wife	Regular	UM	48	20	0.761	0.342	150	1	Male	2.9	H. Wife	Regular	UM	146	25	0.513	0.688
151	3	Male	3	H. Wife	Regular	UM	50	19	0.796	0.544	151	3	Female	3.2	Service	Regular	UM	192	24	0.497	0.682
152	4	Female	3.1	H. Wife	Regular	UM	39	20	0.695	0.448	152	1	Male	2.8	H. Wife	Regular	UM	149	26	0.477	0.79
153	2	Male	2.8	H. Wife	Regular	UP	41	18	0.681	0.412	153	2	Female	1.8	H. Wife	Regular	LM	170	23	0.549	0.701
154	1	Male	3.2	H. Wife	Regular	UM	48	21	0.628	0.425	154	1	Female	3.2	H. Wife	Regular	UM	171	21	0.451	0.701
155	4	Male	2.6	H. Wife	Regular	UM	43	22	0.599	0.406	155	2	Female	2.6	H. Wife	Regular	UM	147	21	0.542	0.72
156	3	Female	2.2	H. Wife	Regular	UM	51	19	0.797	0.582	156	18	Male	2.2	H. Wife	Regular	UM	198	24	0.516	0.713
157	5	Male	2.6	H. Wife	Regular	LM	39	18	0.708	0.33	157	3	Female	2.6	Service	Irregular	UP	184	25	0.477	0.682
158	2	Female	2.9	Service	Regular	LM	37	19	0.628	0.348	158	2	Female	2.9	H. Wife	Regular	UM	207	24	0.51	0.783
159	3	Male	3	H. Wife	Regular	LO	42	22	0.599	0.489	159	3	Male	2,4	H. Wife	Regular	UP	190	25	0.51	0.745
160	2	Male	2.8	H. Wife	Regular	LM	48	19	0.697	0.582	160	4	Female	2.8	H. Wife	Regular	UM	198	19	0.523	0.682
161	1	Male	2.5	H. Wife	Regular	UM	39	18	0.708	0.44	161	3	Male	2.5	H. Wife	Regular	UM	182	24	0.562	0.637
162	3	Male	2.7	H. Wife	Irregular	UM	38	19	0.655	0.349	162	3	Male	2.6	H. Wife	Regular	UM	190	24	0.51	0.49
163	6	Male	2.7	Service	Regular	LM	26	19	0.681	0.605	163	1	Female	2.9	Service	Regular	UM	160	23	0.484	0.701
164	1	Female	2.9	H. Wife	Regular	LM	32	20	0.68	0.382	164	1	Male	2.5	H. Wife	Regular	LM	151	24	0.51	0.484
165	10	Male	2.6	H. Wife	Regular	UM	34	18	0.556	0.401	165	12	Female	2.6	H. Wife	Regular	UM	180	25	0.51	0.771
166	1	Female	2.6	H. Wife	Regular	UP	29	18	0.627	0.516	166	2	Male	2.8	H. Wife	Regular	UM	180	26	0.471	0.809
167	1	Female	2.8	H. Wife	Regular	UM	39	17	0.556	0.567	167	1	Male	3.1	H. Wife	Regular	UM	149	22	0.477	0.688
168	2	Female	2.9	H. Wife	Regular	UM	23	18	0.627	0.586	168	2	Male	2.7	Service	Regular	LM	178	24	0.523	0.726
169	1	Female	3.4	H. Wife	Regular	LM	26	17	0.915	0.561	169	3	Male	2.9	H. Wife	Regular	LM	191	25	0.529	0.79
170	4	Male	2.6	H. Wife	Regular	UM	29	19	0.719	0.395	170	4	Female	3.1	H. Wife	Regular	UM	193	26	0.49	0.777
171	1	Female	2.5	H. Wife	Regular	UM	29	18	0.51	0.61	171	1	Male	1.8	H. Wife	Regular	UM	140	21	0.542	0.682
172	5	Female	2.8	H. Wife	Regular	UM	29	20	0.641	0.442	172	1	Male	3.1	Service	Regular	UM	140	23	0.484	0.662
173	1	Female	2.9	H. Wife	Regular	UM	26	17	0.961	0.618	173	1	Male	2.9	H. Wife	Regular	UM	149	22	0.51	0.618
174	1	Female	2.8	H. Wife	Regular	UM	28	18	0.693	0.605	174	1	Male	2.8	H. Wife	Regular	UM	200	25	0.49	0.688
175	1	Male	3.1	H. Wife	Regular	UM	30	19	0.529	0.369	175	1	Male	3.1	H. Wife	Regular	UM	148	22	0.549	0.611
176	5	Female	3.2	H. Wife	Regular	UM	24	18	0.542	0.357	176	1	Female	1.8	Service	Irregular	UP	140	20	0.477	0.65
177	1	Female	2.8	H. Wife	Regular	UM	32	19	0.667	0.423	177	2	Male	3.2	H. Wife	Regular	UM	192	26	0.484	0.682
178	1	Female	2.9	Service	Regular	UM	27	17	0.549	0.378	178	1	Female	2.6	H. Wife	Regular	UM	156	19	0.523	0.637

179	4	Female	2.5	H. Wife	Regular	UM	30	18	0.621	0.338	179	1	Male	2.2	H. Wife	Regular	UM	149	20	0.464	0.675
180	1	Male	2.8	H. Wife	Regular	UM	27	20	0.712	0.503	180	3	Male	2.3	H. Wife	Regular	UM	204	25	0.477	0.815
181	1	Female	2.9	Service	Regular	UM	26	17	0.719	0.637	181	3	Male	2.9	H. Wife	Irregular	UM	169	24	0.49	0.745
182	2	Female	3.5	H. Wife	Regular	UP	29	18	0.712	0.331	182	5	Female	2.5	H. Wife	Regular	UM	207	25	0.477	0.783
183	6	Female	2.8	H. Wife	Regular	UM	29	18	0.588	0.38	183	11	Male	2.8	Service	Regular	UM	190	27	0.458	0.796
184	1	Female	2.6	H. Wife	Regular	UM	31	19	0.641	0.369	184	12	Female	2.5	H. Wife	Regular	LM	201	24	0.471	0.726
185	1	Male	2.8	H. Wife	Regular	LM	28	18	0.549	0.433	185	1	Female	2.7	H. Wife	Regular	UM	148	23	0.503	0.682
186	4	Female	3.1	H. Wife	Regular	UM	27	19	0.928	0.439	186	1	Female	2.9	H. Wife	Regular	UM	144	22	0.497	0.713
187	7	Female	2.6	H. Wife	Regular	UM	26	17	0.765	0.357	187	3	Female	2.6	Service	Regular	UM	199	24	0.503	0.745
188	6	Male	2.9	H. Wife	Regular	UM	39	19	0.797	0.478	188	2	Male	2.6	H. Wife	Regular	UM	168	27	0.497	0.847
189	1	Female	3.2	H. Wife	Regular	LM	27	18	0.523	0.442	189	1	Male	2.4	H. Wife	Regular	UM	144	18	0.562	0.618
190	1	Male	2.9	H. Wife	Irregular	LM	26	17	0.692	0.535	190	3	Male	3.1	H. Wife	Regular	UM	184	25	0.497	0.675
191	1	Male	2.8	H. Wife	Regular	LO	27	18	0.778	0.605	191	4	Male	2.7	H. Wife	Regular	UP	196	26	0.484	0.764
192	1	Female	2.6	Service	Regular	UM	26	18	0.771	0.414	192	1	Female	2.9	H. Wife	Regular	UM	170	25	0.489	0.678
193	1	Female	2.7	H. Wife	Regular	UM	28	17	0.673	0.565	193	1	Male	3.2	H. Wife	Regular	UM	166	26	0.49	0.671
194	1	Female	2.5	H. Wife	Regular	UM	26	17	0.694	0.399	194	1	Male	3.1	H. Wife	Regular	LM	158	24	0.504	0.65
195	1	Male	3.3	H. Wife	Regular	LM	26	18	0.68	0.58	195	1	Male	2.6	Service	Regular	UP	159	19	0.497	0.679
196	1	Female	3.2	Service	Regular	UP	31	17	0.627	0.538	196	4	Male	2.2	H. Wife	Regular	UM	200	25	0.483	0.71
197	1	Male	2.8	H. Wife	Regular	UM	28	20	0.699	0.452	197	3	Male	2.6	Service	Regular	UM	182	24	0.488	0.697
198	1	Male	2.9	H. Wife	Regular	UM	27	18	0.876	0.393	198	2	Female	2.6	H. Wife	Regular	UM	163	20	0.51	0.69
199	1	Male	3.1	H. Wife	Irregular	UM	28	17	0.549	0.362	199	1	Female	2.3	H. Wife	Regular	LO	156	22	0.52	0.681
200	5	Female	2.7	H. Wife	Regular	UM	38	18	0.686	0.393	200	1	Male	2.8	H. Wife	Regular	UM	154	22	0.53	0.68
201	1	Male	3.5	H. Wife	Regular	UM	25	19	0.601	0.478	201	3	Male	2.5	H. Wife	Regular	UM	184	26	0.479	0.708
202	1	Male	3.2	Service	Regular	LM	26	17	0.582	0.399	202	3	Female	2.7	H. Wife	Regular	UP	163	25	0.498	0.692
203	1	Male	2.9	H. Wife	Regular	UM	28	18	0.758	0.497	203	1	Male	2.9	Service	Regular	UP	152	19	0.505	0.674
204	4	Male	3.3	H. Wife	Regular	UM	25	19	0.795	0.505	204	1	Male	2.6	H. Wife	Regular	UM	155	24	0.49	0.672
205	1	Female	2.6	H. Wife	Regular	UM	28	17	0.706	0.505	205	1	Male	2.5	H. Wife	Regular	UM	158	22	0.53	0.669
206	2	Female	2.5	H. Wife	Regular	UM	20	16	0.673	0.59	206	1	Male	3.1	H. Wife	Regular	UM	149	23	0.521	0.662
207	4	Male	2.9	H. Wife	Regular	UM	30	18	0.601	0.344	207	3	Female	2.7	Service	Regular	UM	184	25	0.481	0.724
208	2	Male	3.2	Service	Regular	UM	31	18	0.667	0.374	208	3	Male	2.9	H. Wife	Regular	UM	176	24	0.504	0.678
209	1	Male	2.4	H. Wife	Regular	UM	28	17	0.601	0.348	209	1	Male	3.2	H. Wife	Regular	UM	150	24	0.499	0.694
210	4	Male	2.7	H. Wife	Regular	UM	27	17	0.542	0.382	210	1	Male	2.6	H. Wife	Regular	UM	147	23	0.55	0.661
211	10	Female	3.2	H. Wife	Regular	UM	30	18	0.529	0.385	211	4	Female	2.2	H. Wife	Regular	LM	189	26	0.472	0.722
212	1	Female	3.2	Service	Regular	LM	28	17	0.595	0.336	212	1	Male	2.6	H. Wife	Regular	UM	154	23	0.56	0.679
213	1	Female	2.7	H. Wife	Regular	UP	33	18	0.536	0.399	213	3	Male	2.9	Service	Regular	UM	185	26	0.475	0.71
214	5	Female	2.8	H. Wife	Regular	LM	29	17	0.627	0.312	214	1	Male	2.2	H. Wife	Regular	UM	159	24	0.522	0.698
215	1	Male	2.5	H. Wife	Regular	LM	28	17	0.673	0.382	215	2	Male	2.8	H. Wife	Irregular	UM	168	24	0.498	0.679
216	1	Male	2.6	H. Wife	Regular	UM	29	22	0.756	0.524	216	1	Female	2.4	H. Wife	Regular	UM	163	25	0.478	0.683

217	1	Female	2.5	H. Wife	Regular	UM	32	18	0.778	0.529	217	2	Female	2.6	Service	Regular	LM	183	25	0.48	0.731
218	5	Female	2.5	Service	Regular	UM	27	17	0.745	0.474	218	1	Male	2.7	H. Wife	Regular	LM	162	24	0.494	0.691
219	1	Female	2.8	H. Wife	Regular	LO	30	18	0.802	0.471	219	1	Male	2.5	H. Wife	Regular	UM	164	24	0.499	0.688
220	13	Female	2.6	H. Wife	Regular	UM	35	17	0.765	0.541	220	2	Male	2.2	H. Wife	Regular	LM	170	25	0.502	0.695
221	1	Male	2.5	H. Wife	Regular	LM	31	18	0.68	0.376	221	3	Male	2.4	H. Wife	Regular	LO	176	26	0.482	0.673
222	8	Female	2.9	H. Wife	Regular	UM	29	19	0.66	0.505	222	2	Female	2.8	H. Wife	Regular	UM	165	25	0.471	0.668
223	13	Male	3.5	H. Wife	Regular	UM	26	20	0.51	0.518	223	5	Female	2.2	H. Wife	Regular	UM	168	26	0.463	0.672
224	1	Female	3.1	H. Wife	Regular	UM	25	18	0.797	0.338	224	5	Female	2.5	H. Wife	Regular	UM	175	25	0.459	0.694
225	2	Male	3.2	H. Wife	Regular	UM	28	17	0.66	0.439	225	2	Male	2.4	H. Wife	Regular	UM	172	26	0.513	0.67
226	1	Female	2.6	Service	Regular	UM	31	18	0.549	0.451	226	6	Female	2.8	Service	Regular	UP	169	24	0.457	0.712
227	1	Male	2.7	H. Wife	Regular	LM	29	20	0.601	0.369	227	2	Male	1.9	H. Wife	Regular	LM	180	25	0.464	0.698
228	9	Female	2.9	H. Wife	Regular	UM	40	19	0.586	0.65	228	1	Male	2.6	H. Wife	Regular	UM	164	26	0.449	0.811
229	1	Male	2.7	H. Wife	Regular	UM	30	17	0.797	0.529	229	3	Female	2.9	Service	Regular	UP	192	23	0.448	0.778
230	1	Male	2.6	H. Wife	Regular	UM	26	18	0.66	0.381	230	1	Male	2.2	H. Wife	Regular	UM	161	21	0.51	0.82
231	12	Male	3.2	H. Wife	Regular	UM	32	18	0.763	0.522	231	2	Male	2.3	H. Wife	Regular	LM	169	25	0.512	0.666
232	1	Male	3.3	Service	Regular	UM	38	19	0.765	0.541	232	1	Female	2.2	H. Wife	Irregular	UM	171	24	0.532	0.842
233	7	Male	2.7	H. Wife	Irregular	UM	30	20	0.68	0.376	233	5	Male	2.8	H. Wife	Regular	UM	168	27	0.5	0.778
234	10	Female	2.8	H. Wife	Regular	UP	31	17	0.66	0.605	234	4	Female	2.5	Service	Regular	UM	170	24	0.511	0.803
235	11	Male	2.6	H. Wife	Regular	LM	32	18	0.577	0.598	235	5	Male	2.6	H. Wife	Regular	UM	185	26	0.471	0.791
236	1	Female	2.9	H. Wife	Regular	UM	30	17	0.797	0.338	236	3	Male	2.9	H. Wife	Regular	LM	182	26	0.499	0.79
237	1	Female	2.5	H. Wife	Regular	LM	36	21	0.66	0.439	237	1	Female	2.8	H. Wife	Regular	LM	169	24	0.514	0.802
238	3	Male	2.6	Service	Regular	UM	27	17	0.549	0.423	238	1	Male	2.5	H. Wife	Regular	LM	172	23	0.489	0.689
239	1	Female	2.7	H. Wife	Regular	LM	36	18	0.601	0.369	239	2	Male	3.1	H. Wife	Regular	UM	185	26	0.559	0.812
240	1	Female	2.8	H. Wife	Regular	UM	37	17	0.565	0.551	240	1	Male	2.4	H. Wife	Regular	UM	187	24	0.492	0.861
241	1	Male	3.5	H. Wife	Regular	UM	35	18	0.797	0.529	241	1	Female	2.3	H. Wife	Regular	UM	160	24	0.55	0.801
242	3	Female	3.2	Service	Regular	UM	40	19	0.66	0.412	242	4	Male	2.4	H. Wife	Regular	UM	175	25	0.454	0.678
243	2	Male	2.5	H. Wife	Regular	LM	33	17	0.777	0.522	243	3	Female	2.5	H. Wife	Regular	LM	177	22	0.479	0.881
244	1	Female	2.7	H. Wife	Regular	UM	27	18	0.68	0.376	244	2	Male	2.3	Service	Regular	UP	190	24	0.509	0.87
245	1	Male	2.8	H. Wife	Regular	UP	30	19	0.66	0.605	245	5	Male	2.6	H. Wife	Regular	UM	179	23	0.491	0.794
246	1	Female	2.9	H. Wife	Regular	UM	36	18	0.51	0.598	246	6	Female	2.7	H. Wife	Regular	UM	211	26	0.492	0.911
247	1	Female	3.4	Service	Regular	UM	28	17	0.797	0.338	247	3	Male	2.5	H. Wife	Irregular	UM	166	26	0.467	0.84
248	13	Female	3.3	H. Wife	Regular	LM	29	18	0.66	0.439	248	3	Female	2.3	H. Wife	Regular	UM	172	21	0.466	0.821
249	1	Female	3.1	H. Wife	Regular	UM	30	19	0.549	0.444	249	2	Male	2.2	H. Wife	Regular	LM	168	22	0.475	0.822
250	1	Female	2.8	H. Wife	Regular	LO	27	17	0.601	0.369	250	4	Male	2.4	H. Wife	Regular	UM	179	24	0.487	0.828
251	1	Male	2.7	H. Wife	Regular	LM	26	21	0.575	0.561	251	2	Male	2.4	H. Wife	Regular	UM	180	24	0.48	0.749
252	10	Female	2.9	H. Wife	Regular	UM	37	17	0.797	0.529	252	2	Female	2.5	Service	Regular	LM	168	26	0.506	0.809
253	2	Female	3.3	H. Wife	Regular	UM	39	20	0.66	0.414	253	3	Female	3.2	H. Wife	Regular	LM	202	25	0.483	0.904
254	1	Male	2.5	H. Wife	Regular	LM	35	19	0.792	0.522	254	2	Male	2.6	H. Wife	Regular	UP	165	24	0.496	0.849

255	1	Female	2.8	Service	Regular	UM	29	18	0.797	0.529	255	3	Male	3.1	H. Wife	Regular	UM	180	26	0.455	0.749
256	1	Male	2.9	H. Wife	Regular	UP	26	17	0.66	0.439	256	15	Female	2.4	H. Wife	Irregular	UM	168	24	0.573	0.779
257	3	Female	2.6	H. Wife	Regular	UM	33	18	0.795	0.43	257	5	Female	2.5	H. Wife	Regular	UM	170	26	0.515	0.94
258	1	Male	3.1	H. Wife	Regular	LM	26	19	0.833	0.449	258	3	Male	2.4	H. Wife	Regular	UM	169	24	0.507	0.777
259	1	Female	2.5	Service	Regular	UM	22	20	0.799	0.511	259	1	Male	2.6	H. Wife	Regular	LM	171	23	0.488	0.679
260	9	Male	2.5	H. Wife	Regular	UM	36	19	0.828	0.49	260	11	Female	2.7	Service	Regular	UP	176	26	0.517	0.689
261	1	Male	2.8	H. Wife	Regular	LM	37	18	0.798	0.446	261	3	Female	2.4	H. Wife	Regular	UM	172	25	0.455	0.871
262	1	Female	2.9	H. Wife	Regular	UM	27	21	0.812	0.414	262	8	Female	2.4	H. Wife	Regular	UM	170	25	0.457	0.877
263	1	Male	3.4	H. Wife	Regular	UM	30	18	0.796	0.547	263	1	Female	2.3	H. Wife	Regular	LM	168	24	0.548	0.668
264	6	Male	2.8	H. Wife	Regular	UM	35	19	0.785	0.578	264	5	Male	2.3	H. Wife	Regular	UM	161	24	0.501	0.699
265	1	Male	3.3	H. Wife	Irregular	UM	31	21	0.79	0.593	265	3	Female	2.5	Service	Regular	UP	168	25	0.515	0.802
266	3	Male	3.4	H. Wife	Regular	UM	42	20	0.698	0.512	266	8	Male	2.9	H. Wife	Regular	UM	173	25	0.521	0.749
267	1	Male	2.7	H. Wife	Regular	LM	27	18	0.754	0.519	267	2	Female	3.1	H. Wife	Regular	UM	180	26	0.469	0.692
268	3	Male	2.5	Service	Regular	UM	39	17	0.766	0.6	268	2	Female	2.8	Service	Regular	UP	169	23	0.478	0.89
269	8	Female	3.4	H. Wife	Regular	UP	38	20	0.799	0.582	269	16	Male	2.6	H. Wife	Regular	UM	173	25	0.525	0.778
270	4	Male	2.6	H. Wife	Regular	UM	35	18	0.766	0.499	270	9	Male	2.5	H. Wife	Regular	UM	187	26	0.492	0.729
271	1	Male	3.5	Service	Regular	UM	31	19	0.855	0.568	271	3	Male	2.4	H. Wife	Regular	UP	161	23	0.493	0.728
272	3	Male	3.2	H. Wife	Irregular	UM	37	17	0.875	0.605	272	10	Female	2.5	H. Wife	Regular	UM	190	24	0.511	0.714
273	2	Male	2.8	H. Wife	Regular	UM	35	20	0.772	0.579	273	1	Female	2.7	H. Wife	Regular	UM	182	26	0.521	0.831
274	1	Female	2.5	Service	Regular	UM	3.3	19	0.698	0.611	274	6	Female	2.3	H. Wife	Regular	UM	172	25	0.487	0.82
275	2	Male	3.3	H. Wife	Regular	LM	2.9	20	0.686	0.558	275	6	Male	1.8	Service	Regular	UP	179	24	0.514	0.868
											276	2	Female	2.2	H. Wife	Regular	UM	181	25	0.487	0.678
											277	7	Female	2.6	Service	Regular	UM	164	24	0.448	0.799
											278	14	Male	2.4	H. Wife	Regular	UM	180	25	0.51	0.709
											279	6	Male	2.5	H. Wife	Regular	UM	167	23	0.497	0.795
											280	2	Female	2.7	H. Wife	Regular	UM	174	26	0.498	0.697
											281	4	Male	3.2	H. Wife	Regular	UM	170	24	0.496	0.688
											282	2	Male	2.7	H. Wife	Regular	UP	166	22	0.479	0.718
											283	3	Male	2.8	Service	Regular	UM	176	24	0.485	0.668
											284	4	Male	3.2	H. Wife	Regular	UM	191	26	0.499	0.657
											285	3	Female	2.2	H. Wife	Regular	UM	175	24	0.479	0.779
											286	4	Male	2.6	H. Wife	Regular	UM	173	25	0.524	0.899
											287	3	Female	2.8	H. Wife	Irregular	UM	168	23	0.527	0.832
											288	1	Female	3.3	Service	Regular	UM	178	25	0.538	0.679
											289	5	Female	2.6	H. Wife	Regular	UM	171	24	0.499	0.71
											290	7	Female	2.3	H. Wife	Regular	UM	181	25	0.469	0.891
											291	7	Male	2.4	H. Wife	Regular	UM	179	24	0.596	0.734
											292	5	Male	2.6	Service	Regular	UM	187	25	0.538	0.819

											293	5	Male	2.5	H. Wife	Irregular	UM	172	24	0.519	0.878
											294	7	Female	2.5	H. Wife	Regular	UM	184	25	0.459	0.877
											295	7	Female	2.7	H. Wife	Regular	UM	179	24	0.506	0.898
											296	6	Male	2.4	H. Wife	Regular	UM	191	25	0.444	0.811
											297	5	Male	2.3	H. Wife	Regular	UM	173	24	0.47	0.683
											298	2	Female	2.5	H. Wife	Regular	UM	168	23	0.514	0.769
											299	4	Female	2.6	H. Wife	Regular	LM	171	25	0.52	0.91
											300	2	Male	2.8	H. Wife	Regular	UM	187	26	0.498	0.746
											301	3	Male	2.3	Service	Regular	UP	182	27	0.501	0.689
											302	2	Male	2.9	H. Wife	Regular	UM	169	23	0.457	0.8
											303	1	Male	3.3	H. Wife	Regular	UM	165	24	0.496	0.762
											304	3	Male	2.1	H. Wife	Regular	UM	177	26	0.451	0.679
											305	4	Male	2.4	H. Wife	Regular	UM	180	25	0.56	0.769
											306	3	Female	2.2	H. Wife	Regular	UM	181	24	0.508	0.686
											307	1	Female	2.5	H. Wife	Regular	UM	178	25	0.459	0.791
											308	4	Female	3.5	H. Wife	Regular	UM	180	26	0.458	0.788
											309	2	Female	2.7	H. Wife	Regular	UM	174	24	0.511	0.84
											310	6	Female	2.1	H. Wife	Regular	UM	175	25	0.499	0.725
											311	5	Female	2.7	H. Wife	Regular	UM	180	26	0.522	0.672
											312	1	Male	2.5	Service	Regular	UM	169	23	0.476	0.793
											313	6	Female	2.6	H. Wife	Regular	UM	180	25	0.496	0.783
											314	3	Male	2.9	H. Wife	Regular	UM	181	26	0.475	0.843
											315	3	Female	2.6	H. Wife	Regular	LM	190	27	0.486	0.912
											316	6	Female	2.4	Service	Regular	UM	184	25	0.477	0.791
											317	1	Female	2.5	H. Wife	Regular	UM	176	25	0.469	0.785
											318	2	Female	2.6	H. Wife	Regular	UM	188	27	0.47	0.844
											319	2	Male	2.4	H. Wife	Regular	UM	189	28	0.459	0.879

Note: Lower. (LO) : .<10000, Lower middle (LM) : 10001-20000 , Upper middle (UM) : 20001-40000, Upper. (UP) : >40000

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