

**Hypothesis** 

# Study of Serum Copper and Iron in Children with Chronic Liver Diseases

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## Abstract

Trace elements have a major role as both oxidants and antioxidants, promoting and protecting from tissue damage respectively. As the liver has a pivotal role in trace elements metabolism and consequenyly their bioavailability, we aimed to measure serum levels of essential trace elements in children with chronic liver diseases (CLDs) regardless the etiology and to study their correlation with liver function tests. Serum zinc (Zn), copper (Cu) and iron (Fe); together with other trace elements parameters; were measured in 50 children with CLDs using spectrophotometry and their levels were compared with those age and sex matched healthy children as controls. Serum Cu, Fe, ferritin and transferrin saturation (TS) were significantly higher in the CLDs group than that in the control group; while serum Zn and total iron binding capacity (TIBC) were significantly lower in the CLDs than that in the control group. Serum Fe, Cu, TS and ferritin were positively correlated with biochemical parameters of liver damage; while serum Zn and TIBC were negatively correlated with biochemical parameters of liver damage. These results encourage inclusion of serum Zn, Cu and Fe as biomarkers for monitoring the severity of liver damage during routine assessment of children with CLDs. We recommended caution to avoid excess Fe and Cu intake in children with CLDs. Moreover, Zn supplementation could be encouraged in children with CLDs regardless its etiology.

Keywords: Chronic liver disease; Copper; Iron; Zinc

# Introduction

Chronic liver diseases (CLDs) are marked by the gradual destruction of liver tissue over time. CLDs in children are considered an important health problem in Egypt. They have a disastrous effect on health, hence economic potential of affected persons. Many risk factors are associated with liver disorders; these are infectious and environmental toxins. Synergism of more than one factor may enhance the process of liver damage [1-3].

Many evidences indicate that trace elements play important roles in a number of biological processes. Trace elements act through activating or inhibiting enzyme reactions, competing with other elements and metalloproteines for binding sites, affecting the permeability of cell membranes and exert enhancing effects on the progression of some diseases [4].

Copper (Cu) is an essential micronutrient. This mineral functions in diverse processes such as infant growth, bone strength, host defense mechanisms, iron transport, red and white blood cell maturation, myocardial contractility, brain development, and cholesterol and glucose metabolism. Cu is indispensible to the formation of collagen and elastin, as well as the electron transport chain in the mitochondria. Cu exists in two oxidation states,  $Cu^{1+}$  and  $Cu^{2+}$ . As such, like most metals, it can act as a pro-oxidant within the cell. Cu is therefore incorporated into various chaperone proteins, and this prevents oxidative damage to the cell. It is also a key component of two ATPases, ATP7A and ATP7B. These ATPases function in the absorption and excretion of Cu into and out of cells, respectively. Defects in these enzymes lead to the genetic disorders such as Menkes disease (MD, *ATP7A*) and Wilson's disease (WD, *ATP7B*) [5].

Iron (Fe) has several vital functions in the body. Fe can alter between two different oxidation states,  $Fe^{2+}$  and  $Fe^{3+}$ . This ability provides Fe with a precious quality in biochemistry, namely the ability to donate or accept an electron. This ability is the reason for the role of Fe, not only in oxygen transport by hemoglobin which is the main function of Fe in the body, but also in DNA synthesis and energy production. The loss of regulation of Fe metabolism and subsequent development of Fe overload is seen in hereditary hemochromatosis (HHC), a common inherited disorder which may lead to progressive organ dysfunction including cirrhosis, arthritis, hypogonadism, diabetes mellitus and cardiomyopathy [6].

Zinc (Zn), Fe and Cu have been regarded as a prime example of competitive biological interactions between metals with similar chemical and physical properties. Elements with similar physical or chemical properties, act antagonistically to each other biologically. Such metals could compete for binding sites on transporter proteins or on enzymes requiring metals as co-factors. However, Zn which occurs exclusively in only one valency state, Zn<sup>2+</sup>, has a stabilizing function and reputedly protects cell membranes against lipid peroxidation, and sulphydryl groups against oxidation. It plays an important role in maintaining the conformation of proteins, and 'Zn fingers' constitute ubiquitous structural elements in many transcription factors. Excessive Zn supply has been shown to inhibit intestinal absorption, hepatic accumulation and placental transfer of Cu, as well as to induce clinical and biochemical signs of Cu deficiency. Thus, Zn is used effectively in treatment of WD [7].

In spite many studies have been performed for the assessment of trace elements in adults with CLDs [8-14], very few studies have been devoted for children [15,16]. So, we aimed to measure the level of some essential trace elements, including serum copper, iron and zinc in children suffering from chronic liver diseases regardless the etiology, and to study their correlation with biochemical measures of liver damage, comparing them with the results of healthy individuals.

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# **Review of Literature**

## Biochemistry of some trace elements

According to body needs, minerals may be divided into macrominerals (major elements) or micro-minerals (trace elements). Macrominerals are defined as minerals that are required in amounts greater than 100 mg/dl. Major minerals include sodium, potassium, chloride, calcium, magnesium and phosphorus. While, trace elements are those inorganic constituents of the body which are required at less than 100 mg/dl, in other word; it forms less than 0.01% of the body weight. Trace elements include copper, iron, zinc, selenium, molybdenum, fluorine, iodine, manganese and cobalt [17].

Trace elements can be subdivided into three categories:

- (1) Those are essential to human health (including iron, copper, zinc, selenium, manganese, chromium, molybdenum and iodine).
- (2) Those are beneficial but not essential (including fluorine, vanadium, boron and lithium).
- (3) Those are not beneficial and primarily associated with toxic effects (including lead, cadmium, mercury and aluminum) [18].

Trace elements with more than one oxidation state such as Cu and Fe participate in oxidation/reduction activities. They involved with many enzymes of citric acid cycle and electron transport chain. Zn with only one oxidation state (Zn<sup>2+</sup>), serves both structural and catalytic functions of major metabolic pathway including protein and nucleic acid synthesis [19,20].

Trace elements and cell cycle: Proliferation is dependent upon Fe. The highest demand for Fe occurs during the late G1 and S phases of the cell cycle (Figure 1) due to the activity of the Fe requiring enzyme, ribonucleotide reductase (RR). RR is an enzyme that catalyzes the de novo biosynthesis of deoxyribonucleotides essential for deoxyribonucleic acid (DNA) replication, cell cycle progression and cell repair. Fe deprivation inhibits RR activity and results in G1/S arrest [21].

The activities of the major enzymes regulating DNA replication, DNA polymerase, are Zn dependent. DNA polymerase is inhibited by Zn deficiency or Zn chelators and is enhanced by addition of low



concentrations of Zn in vitro. Zn is required for expression of multiple genes regulating mitosis, including thymidine kinase and ornithine decarboxylase [20].

Copper plays a fundamental role in regulating cell growth involved in physiological repair processes such as wound healing and angiogenesis as well as in various pathophysiologies including tumor growth, atherosclerosis, and neuron degenerative diseases. The mammalian antioxidant-1 (Atox1) has acquired nuclear function in addition to its Cu chaperone function in cytosol. Atox1 reported as a novel transcription factor that, when activated by Cu, undergoes nuclear translocation, DNA binding, and thereby contributing to cell proliferation [22].

Trace elements and apoptosis: Apoptosis involves a series of biochemical events leading to cell death. The process of apoptosis is controlled by two mechanisms, known as the death receptor (extrinsic) and mitochondrial (intrinsic) pathway. Several studies using a range of Fe chelators have demonstrated their ability to induce apoptosis [21]. Fe chelators such as triapine and tachpyridine have been shown to induce cell death through the activation of the mitochondrial pathway that is p53 independent [23].

The mitochondrial pathway is triggered by a number of stimuli such as DNA damage, ischemia and oxidative stress. This pathway is initialized with the permeabilization of mitochondrial outer membrane leading to protein release, such as cytochrome c (Cc) and apoptosis inducing factor. The release of Cc leads to the formation of apoptisome then induce apoptosis. Fe chelating agents show great deal to be clinically applied in Fe overload disease and a potential anticancer agent, since over 50% of human tumors contain a functionally defective p53 that reduces sensitivity to commonly used chemotherapeutic agents [24-26].

Zinc has numerous chemical properties advantageous for a role in cytoprotection. It protects macromolecules (e.g., proteins, lipids and DNA) from oxidation and proteolysis. Zn deficiency induces apoptosis through DNA fragmentation, nuclear shrinkage, chromatin condensation and apoptotic body formation [27].

Trace elements and immunity: Immune reactions are dependent many secretory proteins released from lymphocytes and on macrophages which stimulate other branches of immunity. Protein synthesis is impaired in the spleen and the thymus of Fe deficient rats. Moreover, the continuous generation of immune cells in bone marrow in response to antigenic stimulation requires the availability of sufficient Fe and Zn. They are so important in the synthesis of deoxyribonucleotide precursors by RR and for the various nucleotidyl transferase and Zn finger proteins that are required for DNA replication and cell division, respectively [28].

Trace elements can affect the immune function directly by maintaining the activity of a number of enzymes that participates in defense processes. The most evident example is the need of heme Fe in the myeloperoxidase dependent generation of hypochlorous acid, which is a well known microbiocidal factor [29]. Moreover; trace elements are responsible for indirect effect by several ways:

(1) Regulating plasma levels of hormones that regulate the development and function of host defense cells. Increased plasma glucocorticoid level and decreased cellular Zn content in Zn deficient animals contributes to a reduction of B, T cells and can result in certain physiologic changes, such as thymic atrophy.

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- (2) Regulating inflammatory cell functions, so Cu deficiency can affect phagocytic cells as it decreases numbers of neutrophils and macrophages. Functions of neutrophils include traveling to the site of infection where they are involved in phagocytosis and killing of foreign invaders. Fe deficiency delayed hypersensitivity reaction, also affect the ability of neutrophils to kill bacteria.
- (3) Regulating the activity of immune cells by synthesis and secretion of cytokines. Cytokines are soluble mediators of immunity secreted by activated immune cells. They are considered as accessory molecules which transmit immune messages. Zn plays an important role in maintaining the proper balance between cells mediated and humoral immunity by regulating patterns of cytokine secretion [30,31].

**Trace elements and production of oxygen radicals:** Cells continuously produce reactive oxygen species (ROS) as a side product of electron transfer during metabolic reactions. There are several major role species resulting from the partial reduction of molecular oxygen  $(O_2)$  as superoxide anion  $(O_2^{-1})$  and hydrogen peroxide  $(H_2O_2)$  as hydroxyl radical (OH). Both (OH) and  $(O_2^{-1})$  are free radicals, since they can abstract an electron from another molecule to achieve a more stable state as they have one unpaired electron in the outer orbital. The excessive generation of free radicals, can result in a state called oxidative stress, which is characterized by a disturbance in the balance between ROS production on one hand and ROS removal with repair of damaged complex molecules on the other hand [32].

The major source of ROS production in the cell is the mitochondrial respiratory chain, which utilizes approximately 80-90% of  $O_2$  which a person consumes. It has been estimated that about 2-3% of  $O_2$  consumed by the respiratory chain is converted to ROS [33].

Another major source of ROS, especially in the liver, is a group of Fe containing enzymes called the cytochrome P450, which are important for metabolizing substances that naturally occur in the body, such as fatty acids, cholesterol, steroids and bile acids [18].

Reactive oxygen species in the body can be produced by two types of immune cells called macrophages and neutrophils. Those contain a group of enzymes called reduced nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase complex, which, when activated, generates  $O_2$  and  $H_2O_2$ .  $H_2O_2$  then interacts with chloride ions present in the cells to produce hypochlorite radical (the active ingredient in bleach), which in turn destroys the pathogen. Zn is an inhibitor of these enzymes [20].

Under normal physiological condition, direct interaction between  $O_2$  and  $H_2O_2$  is not likely to play a significant role in generating OH. However, in the presence of certain metals, particularly free Fe or Cu ions, a sequence of steps leading to the introduction of oxidized group, during the so called Fenton reaction [32,34].

**Trace elements and fenton reaction:** In 1894, Fenton discovered that several metals have a special oxygen transfer property which improves the use of  $H_2O_2$ . Actually, some metals have a strong catalytic power to generate highly reactive OH. Fenton reaction is very important in biological systems as it leads to oxidative damage to proteins, lipids and DNA [35].

# The procedure requires the following:

(1) The typical range for Fe dose is 1 part of Fe per 5-25 parts of  $H_2O_2$ .

(2) The optimal pH occurs between 3 and 6. If the pH is too high then Fe precipitate in the form of Fe  $(OH)_3$  and  $H_2O_2$  will decompose to  $O_2$ .

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- (3) Ferrous sulfate (FeSO<sub>4</sub>) which acts as Fe catalyst contains residual sulfuric acid ( $H_2SO_4$ ) and the  $H_2O_2$  addition is responsible for the fragmentation of organic materials into organic acids.
- (4) H<sub>2</sub>O<sub>2</sub> slowly adding in order to control the increasing of pH and temperature [36].

In fact the availability of ferrous ions (Fe<sup>2+</sup>) limits the rate of reaction, but the recycling of Fe from the ferric (Fe<sup>3+</sup>) to (Fe<sup>2+</sup>) form by a reducing agent can maintain an ongoing Fenton reaction leading to the generation of OH. One suitable reducing agent is  $O_2^-$  which participates in the following reactions in Figure 2:

In Fenton Reaction,  $H_2O_2$  which is found by endogenous metabolism or is available exogenously can produce OH by removing an electron from the metal ion. In Haber Weiss Reaction involving  $O_2^-$  as reducing agent; and the product of auto oxidation of the original metal ions are regenerated and again available for reaction with  $H_2O_2$ . Other transition metals like copper, manganese and cobalt are to catalyze this reaction by cycling between oxidized and reduced states [37,38].

**Trace elements as constituents of antioxidative proteins:** The human body is under attack by ROS. It is known that free metallic ions, (i.e., those not bound to protein molecules or other chelating compounds); catalyze the formation of free radicals in the Fenton reaction [18].

Copper and Zn are part of metallothionein (MT) (Figure 3) – small molecular proteins with a recognized antioxidative function. They have a single chain of 61 aminoacids (6-7 kDa) containing 20 residues of cystein (Cys) appearing in repeating sequences: Cys-X-Cys, Cys-X-Y-Cys or Cys-Cys. Cys is a regular structural unit of MT in all animal species [39].

The electrophilic character of sulfur (S) in the sulfhydryl groups of amino acid is responsible for its high affinity to metallic ions. MT displays the highest affinity for metals of the transitory groups (zinc, copper, cadmium and silver). One molecule of protein can bind 7 atoms of bivalent metals (e.g. zinc) or a larger amount (12 atoms) of univalent metals (e.g. silver). The chief function of MT is to bind





and distribute Zn and Cu, and in case of environmental pollution to bind toxic metals. Several lines of evidence suggest a role for MT in treatment of malignant tumors, regulation of blood pressure and protection against some neurological diseases [40].

Catalytic defense is carried out by enzymes which have the role of chelating potentially toxic transition metals. These enzymes are superoxide dismutase (SOD) and catalase. SOD catalyzes the rapid removal of  $O_2^-$ . This enzyme is organized as a homodimer, presenting two equivalents of Cu per mole of enzyme, together with two equivalents of Zn, which is catalytically inert.

SOD  $(Cu^{+1}/Zn) + O_2^{-1} + 2 H^+ \rightarrow SOD (Cu^{+2}/Zn) + H_2O_2^{-1}$ 

In mammals there are several types of SOD, which differ with respect to their location in the cells and the metal ions they require for their function. SOD (Cu/Zn) is present in the cytosol and in the space between the two membranes surrounding the mitochondria. Manganese containing SOD is present in the mitochondrial matrix. Another form exclusively present in microbial dismutase identified as an iron protein [34,41].

Catalase is Fe containing enzyme found primarily in the small membrane enclosed cell components called peroxisomes. One way that catalase acts is by catalyzing a reaction between two  $H_2O_2$  molecules, resulting in the formation of water and oxygen [42].

$$2 H_2O_2 \rightarrow O_2 + 2 H_2O_2$$

# Copper (Cu)

Copper is an essential nutrient; that plays a key role in many biological processes. Cu is involved in numerous physiological functions: in the normal function of the nervous system and the cardiovascular system, to help transport Fe and to protect cells against destruction by oxidation. Cu is also essential for bone growth and strength, as well as for a healthy immune system. Human health is at more risk from a lack of Cu, than from its excess. A minor decrease in Cu often leads to reduce resistance to infections, fertility problems, chronic fatigue and weakness. Moreover, Cu deficiency can cause anemia, neutropenia, osteoporosis, loss of pigmentation, neurological symptoms and impaired growth [43].

**Copper dietary recommendations:** The most well known form of dietary recommendation is the recommended daily allowance, or RDA. Initially established in 1941 by the Food and Nutrition Board of the US Institute of Medicine, the RDAs are updated periodically to reflect more current researches. The RDA system has largely been replaced by the use of dietary reference intakes, or DRIs. The DRIs represent the most current set of recommendations by the Food and Nutrition Board. In 2001, the Food and Nutrition Board issued a DRI for Cu as represented in Table 1 [44].

## Copper metabolism:

Absorption and distribution: Copper is absorbed from the stomach to some extent, but the major site of absorption is the duodenum. The pH of the stomach will dissociate many weak Cu complexes. Low molecular weight substances (e.g. amino acids) in gastrointestinal secretions such as saliva, gastric and pancreatic juice, bind Cu and thereby maintain the metal in solution in the alkaline milieu of the upper small intestine. Moreover, it has been suggested that Cu is primarily absorbed in the form of amino acid complexes as seen in Figure 4 [45]. The liver is the major organ for the distribution of Cu in mammals. The liver sequesters the newly absorbed Cu, routing it through the blood to other tissues [46].

**Transport and storage:** In humans, approximately 80-90% of the plasma Cu is tightly bound to ceruloplasmin (Cp) while the rest is bound to albumin, histidine and amino acids. A little part is not transported into hepatocytes, but it is excreted by the urine. Cu enzymes can not acquire Cu ions directly from a cytoplasmic pool. Cu "taxicabs" are needed to deliver Cu ions to the enzymes that require it. Cu chaperones are a specialized family of proteins used for this task [47].

Copper enters the hepatocytes and traverses the cell via the Cu transport protein; Ctr1. Characterization of Ctr1 confirms its localization on the plasma membrane [48]. Cu, once inside the hepatocytes, has one of four possible fates (Figure 5):

- (1) Joining the Cu/MT pool which regulates the hepatic storage of Cu. At high concentration, Cu can stimulate MT synthesis. Cu is strictly controlled inside the cell due to its toxicity, and therefore no free Cu ions are present in the cell under normal physiological conditions. So, Cu that eludes binding to intestinal MT is transported to the liver bounded to liver MT, from which it is ultimately released into bile and excreted in the feces when the cell is sloughed off [5].
- (2) Trafficking to the mitochondria for cytochrome c oxidase (CcO) synthesis via the copper chaperone Cox17 [49].

Age	Cu (µg/day)
0-6 months	200 µg
6-12 months	220 µg
1-10 years	340 - 700 μg
11- 18 years	700 - 890 µg
19- 50 years	900 µg
> 50 years	900 µg
Pregnant women	1000 µg
Lactating women	1300 µg

 Table 1: Dietary reference intake of copper (Trumbo et al. [44]).

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- (3) Binding to Cu chaperone for SOD (CCS) which delivers copper to either cytoplasmic or mitochondrial SOD, and catalyzes the formation of an essential disulfide bridge in SOD [50].
- (4) Trafficking via Atox-1 to Cu transporting ATP-ase (ATP7B in hepatocyte and ATP7A in other cells), an enzyme found in trans-Golgi network (TGN). These proteins are part of the P-type ATPase family, a group of proteins that transport metals into and out of cells by using energy stored in the molecule adenosine triphosphate (ATP). Localization studies on ATPase reveal its redistribution from the TGN to a vesicular compartment that moves out toward biliary epithelium under conditions of high Cu concentration, providing a mechanism for Cu excretion in the bile then elimination in the feces. Mutations in *ATP7A* or *ATP7B* respectively disrupt Cu

homeostatic balance, resulting in Cu deficiency (Menkes disease, MD) or Cu overload (Wilson's disease, WD) [47].

**Ceruloplasmin (Cp):** Ceruloplasmin is a blue glycoprotein, synthesized in the liver, containing 6 atom of Cu in its structure. The molecular weight of human Cp is reported to be 151 kDa. Cp carries 90% of Cu in our plasma. The other 10% is carried by albumin, which has a greater importance as it binds Cu less tightly than Cp [51]. The function of Cu seems to be related to its ability to engage in oxidation/ reduction reactions;  $Cu^{+2} \leftrightarrow Cu^+$ . Cp is an important ferroxidase that facilitates the release of Fe from Fe stores by oxidizing Fe<sup>2+</sup> to Fe<sup>3+</sup> prior to its incorporation into plasma transferrin, (i.e., transferrin can only carry Fe<sup>3+</sup>) (Figure 6). Mutation in the *Cp* gene can lead to the rare genetic human disease aceruloplasminemia, characterized by Fe overload in the brain, liver, pancreas and retina [52].



Ceruloplasmin and hephaestin exhibits a copper-dependent oxidase activity, which is associated with possible oxidation of  $Fe^{2+}$  (ferrous iron) into  $Fe^{3+}$  (ferric iron). Abbreviation: Cp=ceruloplasmin, Hp=hephaestin, Fp=ferroportin, Fe<sub>2</sub>-Tf=diferric transferrin, FeR=ferric reductase and DMT1=divalent metal transporter, A=the iron cycle, B=macrophage and C=duodenal enterocyte.

Figure 6: Role of ceruloplasmin and ceruloplasmin homologue hephaestin in iron metabolism (Hellman and Gitlin [51]).

**Genetic defects in copper metabolism:** Wilson's disease (WD; autosomal recessive) and Menkes disease (MD; X-linked) represent the most recognized and understood disorders of Cu homeostasis.

**Wilson's disease (WD):** Wilson's disease is an autosomal recessive disorder of Cu metabolism, caused by mutations within the *ATP7B* gene which is located on chromosome 13q14.1. *ATP7B* gene causes impaired biliary Cu excretion resulting in hepatic copper toxicity and subsequent multisystem disease involving in liver, brain, cornea, skeleton and rarely the heart. In childhood hepatic manifestations predominate with a highly variable spectrum ranging from self-limiting hepatitis to fulminant hepatic failure [53].

**Pathogenesis of Wilson's disease:** Hepatocytes are the primary site of Cu uptake and accumulation in the liver. They sense the Cu status in the cytoplasm and regulate Cu excretion into the bile depending on the intracellular concentration of this metal. This regulation is accomplished by the protein product of the *ATP7B* gene [54].

Copper transporting P-type protein (ATPase) that transports Cu across cell membranes is ATP7B. The ATP7B protein has two functions: (1) Forming an active Cu channel in TGN to move Cu from

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cytosol to the apo-protein of ceruloplasmin; and (2) Cu export into the bile canaliculus (Figure 7). This explains the two cardinal abnormalities in WD: (1) Failure of ceruloplasmin synthesis; and (2); Failure of Cu excretion into [55].

Copper accumulation might be associated with increased oxidative stress and damage within target tissues where Cu concentrations are high. High intra-mitochondrial Cu concentrations might increase free radical generation. Mitochondrial changes including abnormal morphology have been identified in liver from patients with WD. There is evidence of a 33-fold increase of mitochondrial Cu and of oxidative damage in the liver in these patients. Also, there is significant decrease in mitochondrial enzyme activities in livers of patients with WD as compared with patients with cholestatic liver diseases with high copper accumulation [56]. This implies that oxidative damage and mitochondrial dysfunction are important in the pathogenesis of WD [57].

Clinical manifestations: The presenting features of WD are related to deposition of Cu in specific organs, most commonly the liver and central nervous system. Clinical manifestations can be represented in Table 2.



Figure 7: Schematic representation of Cu metabolism within a liver cell (Roberts and Schilsky [7]).

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➤ **Treatment:** Penicillamine has been the cornerstone of drug treatment for WD since 1954. It initially acts to chelate Cu and hence promote its urinary excretion. It also promotes the sequestration of Cu in an inert complex with MT. Penicillamine also has a direct anti-inflammatory and anti-fibrotic action [58].

Zinc acts to prevent dietary Cu absorption by inducing intestinal mucosal MT synthesis. This results in dietary Cu becoming sequestered in the gut mucosa and subsequently sloughed off with normal turnover of the mucosa. Existing hepatic Cu is detoxified in a similar fashion as penicillamine [7].

Liver transplantation will be indicated in those presenting with liver failure or those with advanced CLDs unresponsive to medical treatment. Neurological disease usually responds to transplantation [55].

**Menkes disease (MD):** Menkes disease is an X-linked (Xq13.3) multisystemic lethal disorder of Cu metabolism. Patients usually exhibit a severe clinical course with death before the third year of life. MD occurs as a mutation in the *ATP7A* gene. ATP7A is energy dependent, trans-membrane protein, which is involved in the delivery of Cu to the secreted Cu enzymes and in the export of surplus Cu from cells [59].

➤ **Copper homeostasis in MD:** Menkes disease is characterized by a misdistribution of Cu among organs and within cells, with low Cu values in liver and brain, and normal or high values in kidneys, pancreas or muscles, although, the high Cu level does not reach a toxic state in MD. This is partly due to already diminished intestinal Cu absorption, because of defective Cu export from the mucosal epithelium, and may be due to the scavenger role of MT [60].

In the liver of MD patients, the low Cu content is due to requirement of the metal in other tissues, rather than disturbed Cu metabolism, as the normal liver ATP7B, but not ATP7A, is the main Cu transporter. The reason for the low Cu content in the brain of MD patients is however different. In MD patients, Cu is likely trapped in both the blood-brain barrier and the blood-cerebrospinal fluid barrier, while the neurons and glial cells are deprived of Cu. This also supports the role of ATP7A in brain Cu uptake. Neuronal demyelination is also observed in MD patients due to ATP7A inactivation [61].

Clinical manifestation: Progressive neurodegeneration and marked connective tissue dysfunction characterize the clinical picture of the most common severe form of MD, and death typically occurs before the third year of life, also note the lax skin in Figure 8.

In the early neonatal period patients may present with prolonged jaundice, hypothermia, hypoglycemia and feeding difficulties. The first sign of MD may be unusual sparse and lusterless scalp hair that becomes tangled on the top of the head at the age of 1–2 months (Figure 9) [62].

Initial psychomotor development is usually unremarkable with normal babbling and smiling up to about 2–4 months of age. The baby then ceases to develop further and gradually loses some of the previously developed skills. Most patients develop therapy resistant seizures from about 2 to 3 months of age. As the motor dysfunction progresses, spontaneous movements become limited, drowsiness and lethargy emerge. The patients are typically diagnosed at 3–6 months of age, often due to the abnormal hair that is a striking feature of the disease [63].

Vascular, urogenital, and skeletal abnormalities are numerous.

Routine ophthalmoscopy is usually normal, but in later stages patients frequently fail to follow visual stimulus. Late manifestations of the disease are blindness, subdural hematoma, and respiratory failure. Most patients die early due to infection, vascular complications (such as sudden and massive cerebral hemorrhage due to vascular rupture), or from the neurological degeneration it self [61].

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➤ **Treatment:** Menkes disease is a progressive disorder leading to death in early childhood in its severe forms, although some patients survive above 5 years of age. However, careful medical care, and possibly Cu administration, may extend life span up to 13 years or even more. Oral administration of Cu is ineffective as Cu is trapped in the intestines; it should be supplemented parenterally or subcutaneously. Among the available Cu compounds, Cu-histidine has proved to be the most effective [64].

## Iron (Fe)

It is doubtful whether any form of life exists in the absence of Fe. Fe is required for many enzymes that are critical for cellular function,

1. Hepatic (usually 4-12 years)	<ul> <li>Asymptomatic hepatomegaly</li> <li>Isolated splenomegaly</li> <li>Persistently elevated liver function tests</li> <li>Fatty liver</li> <li>Acute hepatitis</li> <li>Resembling autoimmune hepatitis</li> <li>Cirrhosis: compensated or decompensated</li> <li>Acute liver failure</li> </ul>
2. Neurological (in second decade)	<ul> <li>Movement disorder</li> <li>Rigid dystonia</li> <li>Dysautonomia</li> <li>Insomnia</li> <li>Dysarthria</li> <li>Seizures</li> </ul>
3. Psychiatric	<ul> <li>Depression</li> <li>Neurotic behaviorous</li> <li>Personality changes</li> <li>Psychosis</li> </ul>
4. Other systems	<ul> <li>Ocular: Kayser-Fleischer rings, sunflower cataracts</li> <li>Renal abnormalities: aminoaciduria, nephrolithiasis</li> <li>Skeletal abnormalities: premature osteoporosis, arthritis Cardiomyopathy</li> <li>Pancreatitis</li> <li>Hypoparathyroidism</li> <li>Infertility, menstrual irregularities, miscarriages</li> </ul>

 Table 2: Modes of presentation of WD (Roberts and Schilsky [7]).



Figure 8: Clinical appearance at age 3 weeks of patient with MD (Tumer and Moller, [59]).



and plays a fundamental role in oxygen carrying proteins such as haemoglobin and myoglobin. Fe can also be toxic when present in excess as it is able to catalyze the formation of ROS. It is so important that highly specialized proteins have been developed for efficient extracellular transport (transferrin) and intracellular storage (ferritin) of Fe [65].

**Body distribution and dietary recommendation:** The distribution of Fe in tissue is shown in Figure 10. More than two thirds of the body Fe content is incorporated into hemoglobin in developing erythroid precursors and mature red cells. Most of the remaining body Fe is found in hepatocytes and reticuloendothelial macrophages, which serve as storage depots. Approximately 10-15% is present in muscle fiber (in myoglobin) and other tissues (in enzymes and cytochromes) [66]. The DRI of Fe is listed in Table 3 [44].

## Iron metabolism:

**Iron absorption:** Under normal circumstances, only 1-2 mg of elemental iron is absorbed per day by duodenal enterocytes, in balance with gastrointestinal and other losses (Figure 10). Humans have no significant excretory pathway for Fe. Thus, body Fe stores are normally controlled at the level of absorption, matching absorption to physiologic requirements. Iron absorbtion is regulated by: (1) Dietary regulator: a short-term increase in dietary iron as the mucosal cells have accumulated iron and "block" additional uptake; (2) Stores regulator: as iron stores increase in the liver, hepcidin is released and diminished intestinal ferroportin release and the enterocytes retain any absorbed iron and are sloughed off in a few days [66].

> Role of duodenal crypt cells in iron absorption (Figure 11): Duodenal crypt cells sense body Fe status and are programmed for Fe absorption as they mature. Duodenal and proximal jejunal enterocytes are responsible for Fe absorption [67]. This process can be explained as follow:

- (a) Low gastric pH helps to dissolve Fe.
- (b) Fe is then enzymatically reduced to the  $Fe^{2+}$  form by ferric reductase.

- (c) Divalent metal transporter 1 (DMT-1) transfers Fe to the enterocyte. DMT-1 levels are altered in response to body Fe stores.
- (e) In enterocyte, Fe is either stored as ferritin or moved across the basolateral membrane to reach the plasma.
- (f) When Fe is exported to plasma from the enterocyte through ferroportin-1 (Fpn1), it is rapidly oxidized to Fe<sup>3+</sup> form by hephaestin and bound to transferrin [68].

Iron must cross two membranes to be transferred across the absorptive epithelium. Each transmembrane transporter is coupled to an enzyme that changes the oxidation state of iron. The apical transporter has been identified as DMT1. It acts in concert with a type of ferric reductase activity. The basolateral transporter which called ferroportin-1 (Fpn1) requires hephaestin, a ceruloplasmin like molecule, for the transfer of iron to the plasma. On the basis of its



Figure 10: Distribution of iron in human body (Andrews [66]).

	Age (years)	Iron (mg/day)
Infants	0.5-3	15
Children	4-10	10
Adolescent Male	11-18	10
Adolescent Female	11-18	18
Adult Men	>18	18
Adult women	19-50	18
	>50	10

 Table 3: Dietary reference intake of iron for age and sex (Trumbo et al. [44]).

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Iron must cross two membranes to be transferred across the absorptive epithelium. Each transmembrane transporter is coupled to an enzyme that changes the oxidation state of iron. The apical transporter has been identified as DMT1. It acts in concert with a type of ferric reductase activity. The basolateral transporter which called ferroportin-1 (Fpn1) requires hephaestin, a ceruloplasmin like molecule, for the transfer of iron to the plasma. On the basis of its structure, hephaestin is presumed to be a form of ferroxidase. Iron within enterocytes is stored as ferritin.

Figure 11: Iron Transport across the Intestinal Epithelium (Andrews [69]).

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➤ Role of hepcidin and ferroportin in iron absorption (Figure 12): Hepcidin (HAMP) an iron regulatory hormone is produced in the liver. Fpn1 is an iron exporter present on the surface of absorptive enterocyte, macrophage, hepatocyte and placental cells. HAMP binds to Fpn1. After binding, Fpn1 internalized and degraded to decrease export of cellular Fe. Homeostasis loop is complete as Fe regulates the secretion of HAMP, which in turn controls the concentration of Fpn1 on the cell surface [70].

During times of low Fe status, HAMP is low and fpn1 expression

on the basolateral membrane of the enterocyte is maintained. As a consequence, intestinal epithelial cells transport more dietary Fe across their basolateral membranes leading to increase Fe absorption [71].

Mutations in both the *HAMP* (juvenile hemochromatosis) and *Fpn1* (autosomal dominant hemochromatosis) genes have been described. HAMP expression is regulated (stimulated) by the HFE protein, transferrin receptor 2 (TfR2) and Hemojuvelin protein (HJV) [72].

This regulation provides a probable link for the mechanism of Fe overload in the various forms of hemochromatosis (Figure 12). In cases with hemochromatosis, the normal increase in HAMP expression with Fe loading is lost, leading to lower HAMP levels and continued Fe absorption in spite of Fe overload [71].

Iron absorption Iron in the circulation (Figures 12 and 13): Iron in the circulation is tightly bound to transferrin (Tf). Tf can bind up to two molecules of  $Fe^{3+}$ , with about 30% of the binding sites on Tf normally occupied by  $Fe^{3+}$ . Diferric transferrin ( $Fe_2$ -Tf) binds to the TfR on the cellular plasma membrane. This complex is then endocytosed into the cell, where Fe is released by the acid environment of the endocytic vesicle. Fe is then transported across the endosomal membrane to the cytoplasm [65].

Cellular iron uptake (Figure 13): The uptake of Fe is primarily



In the duodenal enterocyte, dietary iron is reduced to the ferrous state by duodenal ferric reductase, recently known as duodenal cytochrome b reductase (Dcytb), transported into the cell by divalent metal transporter 1 (DMT1), and released by way of ferroportin into the circulation. Hephaestin facilitates enterocyte iron release. Hepatocytes take up iron from the circulation either as free iron or transferrin-bound iron (through transferrin receptor 1 and transferrin receptor 2). Transferrin receptor 2 may serve as a sensor of circulating transferrin-bound iron, thereby influencing expression of the iron regulatory hormone hepcidin. The hepcidin response is also modulated by HFE and hemojuvelin. Hepcidin is secreted into the circulation, where it down-regulates the ferroportin-mediated release of iron from enterocytes, macrophages, and hepatocytes.

Figure 12: Interplay of key proteins in iron homeostasis (dashed red lines) [71].

regulated by the expression of the TfR on the cell surface as follow:

(1) In Fe deficiency, iron regulatory proteins (IRP) are increased and bind to the iron responsive elements (IRE) in the 3'-untranslated region of the transferrin receptor messenger RNA (mRNA). This stabilizes the transferrin receptor transcript, leading to increased transferrin receptor expression on the cell surface, increasing iron uptake. Simultaneously, these same (IRP-IRE) bind to the 5'-untranslated region of ferritin (the iron storage and intracellular sequestration molecule), decreasing its synthesis.

(2) In states of Fe repletion or excess, there is a reduced level of IRP, leading to less TfR production and an increase in ferritin and HAMP synthesis [73].

Human transferrin is synthesized as a 698 amino acid precursor. Apo-transferrin (iron-free) initially binds iron at the C-terminus, followed by iron binding at the N-terminus to form Holo-transferrin (diferric-transferrin). Holo-transferrin will interact with TfR1 on the surface of cells where it is internalized into acidified endosomes. and mediates the uptake of transferrin bound iron (TBI). This protein is predominately expressed in the liver, where, in contrast to TfR, it is not down regulated by dietary Fe overload or in the mouse model for hereditary hemochromatosis (HHC). Hepatic TfR2 provides an explanation for the continued hepatic Fe uptake in HHC despite the down regulation of TfR [74].

Main actions and factors regulating the expression of the major Fe transporter proteins are summarized in Table 4.

**Iron deficiency (ID):** Iron deficiency is globally the most common form of nutrient deficiency, affecting an estimated two billion people, and causing approximately 0.8 million deaths a year worldwide as reported by world health organization (WHO). Symptoms of ID, are diverse and range from fatigue and decreased aerobic performance, to altered cognitive functions, immune system alterations, increased risk of maternal and child mortality, and thermoregulation disorders [75]. Considering that DMT-1 has the ability to handle other divalent metal ions than Fe; ID leads to increased heavy metal absorption, which gives another dimension to ID as a health problem [76].

Transferrin receptor 2 binds holo-transferrin (diferric-transferrin)

Iron overload disorders: Iron overload disorders represent a



Ferrous iron transporter

Invaginate to form specialized endosomes. A proton pump decreases the pH within the endosomes, leading to conformational changes in proteins that result in the release of iron from transferrin. The iron transporter DMT1 moves iron across the endosomal membrane, to enter the cytoplasm. Meanwhile, transferrin (Apo-Tf) and transferrin receptor are recycled to the cell surface, where each can be used for further cycles of iron binding and iron uptake. In erythroid cells, most iron moves into mitochondria, where it is incorporated into protoporphyrin to make heme. In nonerythroid cells, iron is stored as ferritin and hemosiderin.

Figure 13: The Transferrin Cycle (Andrews [69]).

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heterogenous group of conditions resulting from inherited and acquired causes. With the discovery of new proteins and genetic defects, a greater insight has been gained into their causation at the molecular level and the complex mechanisms of normal and disordered Fe homeostasis [6]. Clinical disorders of Fe overload are classified in Table 5.

Mechanisms of iron overload in hereditary hemochromatosis and juvenile hemochromatosis: In Hereditary hemochromatosis (HHC) (the *HFE* gene located on chromosome 6p21.3), the two most common mutations are designated as C282Y and H63D, the C282Y mutation, a single point mutation with substitution of tyrosine for cysteine at position 282, accounts for most cases of HHC; while juvenile hemochromatosis (JHC) (type 2A: the *HJV* gene is located on chromosome 1q21; type 2B: the *HAMP* gene is located on chromosome 19q13) [77].

Net Fe absorption is increased above endogenous losses; this is directly related to dysregulation of hepatic HAMP expression. The result is a net gradual increase in total body Fe with normal erythropoiesis. Fe is deposited in parenchymal cells of the liver, heart and subgroup of endocrine tissues [78].

In HHC, the net increase in total body Fe has been estimated at 4–7 mg/day. The increased Fe absorption is the result of inappropriate transfer of Fe at the basolateral surface of the enterocyte due to suppression of hepatic HAMP secretion. It is reported that the mutant HFE does not bind to transferrin receptor and so not reduces its affinity for transferrin bound iron (TBI), note that the three genes *-HFE*, *TfR2*, and *HJV-* all encode for proteins that affect HAMP. While in JHC; Fe accumulates more rapidly than in HHC, perhaps related to a more significant role of HJV in the regulation of HAMP [77].

Mechanisms of iron overload in secondary iron overload disorders: In secondary Fe overload due to hemoglobinopathies, Fe overload is related to both: (1) The anemia with increased Fe absorption resulting in an additional 2-5 g of iron absorbed from the diet per year.

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(2) Excess Fe provided by transfusions that bypasses the normal intestinal regulation of Fe absorption.

Fe accumulates in reticuloendothelial macrophages first, and then spills over parenchymal cells. Parenchymal Fe loading is dangerous, leading to tissue damage and fibrosis ending with organ damage [79].

# Zinc (Zn)

Zinc, although present in a minute quantity in humans, is an essential nutrient with three major biological roles, as catalyst, structural, and regulatory ion. It plays an important role as a component of many enzymes regulating cell growth, protein synthesis, energy metabolism, gene transcription, hormone levels, and growth factor metabolism [80,81].

Zn affects growth hormone (GH) metabolism which is the key hormone for growth and development. Zn ion induces the dimerization of GH in the way that two Zn ions associate per dimer of GH in a cooperative fashion. Formation of a Zn-GH dimeric complex may be important for storage of GH in secretary granules [20].

Zinc is reported to influence hemostasis by affecting platelet aggregation and coagulation. Zn enhances thrombin activity of the common coagulation pathway, the acceleration of fibrin polymerisation, platelet activation and the initiation of the intrinsic pathway [82].

Zinc deficiency was a cause of growth retardation and delayed sexual development in men. Zn content of the prostate gland, the seminal fluid and ejaculated sperm are very high and testicular Zn is essential for spermatogenesis. Zn deficiency has also been reported to be a cause of hypogonadism [83].

Body distribution & dietary recommendation: The total body

Transporter Proteins	Location	Action	Regulatory Factors
DMT-1	Duodenal enterocytes (apical membrane)	Iron uptake from gut lumen	Stimulated by low iron concentration in enterocyte cytoplasm
Fpn1	Duodenal enterocytes (basolateral membrane), hepatocytes, macrophages (including kupffer cells)	Iron transport from cell cytoplasm into plasma	Stimulated by high cytoplasmic iron concentration. Inhibited by hepcidin.
TFR1	Duodenal enterocytes (basolateral membrane), hepatocytes	Iron transport from plasma into cell cytoplasm	Stimulated by low cytoplasmic iron levels, hypoxia and Proinflammatory cytokines. Also regulated by HFE (mechanism unknown)
TFR2	Duodenal enterocytes (basolateral membrane), hepatocytes	Iron transport from plasma into cell cytoplasm.Also regulates hepcidin production by hepatocytes	Stimulated by high transferrin levels. Also regulated by HFE (mechanism unknown)

Abbreviation: DMT- 1= divalent metal transporter, Fpn1= ferroportin-1, TFR1= transferrin reseptor-1, TFR2= transferrin reseptor-2.

Table 4: Main actions and factors regulate the major iron transporter proteins (Deugnier et al. [65]).

Familial or hereditary forms of hemochromatosis
Type 1 hereditary hemochromatosis
Type 2 juvenile hemochromatosis
Type 3 transferrin receptor 2 mutations
Type 4 ferroportin mutation
Type 5 ferritin mutation
Aceruloplasminemia
Atransferrinemia
Neonatal iron overload
Secondary iron overload
<ul> <li>Iron loading anemia (sideroblastic anemia, hereditary spherocytosis and β-thalasemia)</li> </ul>
Transfusional iron overload
Iron overload in chronic liver disease

Table 5: Classifications of iron overload disorders (Siah et al. [6]).

content of Zn in a hypothetical 70 Kg man approximates 2.5 gm with 30% of the total Zn present in bone and 60% in muscle. The highest concentrations occur in eyes, hair and male reproductive organs. Intermediate levels are present in liver and kidney. DRI of Zn is represented in Table 6 as recommended by WHO [44].

Zinc absorption & homeostasis: Absorption of Zn occurs mainly in the duodenum and the proximal small intestine, with approximately 60% in the duodenum, 30% in the ileum, 8% in the jejunum, and 2% through the colon [84]. The mechanism probably involves an active transport facilitated by low molecular weight ligands of pancreatic origin. The ligands bind Zn and transport it to the luminal surfaces of the intestinal epithelial cell. From there, Zn is transferred to a binding site on the basolateral membrane, where it becomes available for attachment to its transporter proteins in the portal circulation. Approximately 50% is freely exchangeable, being loosely bound to albumin. Another 7% is amino-acid bound mainly to histidine and cysteine. The remaining plasma Zn is tightly bound to  $\alpha$ -2 macroglobulins, and to other serum proteins. Transferrin can also bind Zn and might play a role in the distribution of Zn in the portal venous system [85].

The small intestine plays a central role in Zn metabolism and the maintenance of whole body Zn homeostasis. Note that, there are no reserve stores of Zn, such as ferritin for Fe. The main route of excretion of Zn occurs in the intestine. Fecal losses of Zn in adults consuming 15 mg of dietary Zn is approximate 10 mg/day. Urinary losses of zinc are between 0.3 and 0.5 mg/24 hrs, primarily as amino-acid bound Zn with some porphyrin bound Zn [84].

**Mammalian zinc transporters:** Mammalian Zn transporters are within two gene families [86]:

- i. The ZnT family [solute-linked carrier 30 (SLC30)]
- ii. The Zip family [solute-linked carrier 39 (SLC39)]

Zinc transporters families appear to have opposite roles in cellular Zn homeostasis, where ZnT transporters reduce intracellular cytoplasmic Zn by promoting Zn efflux from cells or into intracellular vesicles, while Zip transporters increase intracellular cytoplasmic Zn by promoting extracellular and, perhaps, vesicular Zn transport into cytoplasm. However, mechanisms of transport by these families are still not well characterized. Furthermore, most ZnT and Zip families show evidence of polymorphisms, which could produce structurally different proteins and, hence, could influence dietary Zn requirements and Zn metabolism [87].

**ZnT (SLC30) family:** More than 100 members of the SLC30 family are found in organisms at all phylogenetic levels. This family is divided into three subfamilies:

- (1) Subfamily I contains a large proportion of prokaryotic members.
- (2) Subfamilies II and III contain eukaryotic and prokaryotic members in a similar proportion.

Most ZnT family has six transmembrane domains (TMDs). ZnT family has a long histidine rich loop between TMDs IV and V, which could represent a metal-binding domain. Deletion of TMDs I and II or the C terminus of ZnT1 disrupts transporter activity; therefore these domains appear critical for Zn transport [88].

**ZIP (SLC39) family:** There are at least 86 members of the Zip family. Zip proteins have been divided into two subfamilies [89]:

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- (1) Subfamily I consist mostly of fungal and plant sequences.
- (2) Subfamily II is composed of insect, nematode, and mammalian sequences.

Most Zip family is predicted to have eight TMDs with extracellular (or intravascular) amino and carboxyl termini. A common feature among ZIP family is a long loop region of histidine between TMDs III and IV and a very short C terminus. The greatest degree of conservation in the Zip family is found in TMD IV to VIII [90].

**Effect of zinc on liver:** Patients with liver diseases may become Zn depleted through inadequate dietary intake, impaired absorption, or increased clearance of Zn. Zn administration has been shown to inhibit the accumulation of hepatic collagen in experimentally produced hepatic necrosis and to significantly improve neurologic signs in hepatic encephalopathy in humans [91].

Zinc induction of the Cu binding protein (metallothionein, MT) in the gastrointestinal tract and hepatocyte assists in the trapping and elimination of injurious Cu. Induction of MT in the hepatocyte also is believed to protect against lysosomal Cu loading and subsequent cell autolysis. The pathologic effects of accumulated Fe in hepatic macrophages in necroinflammatory liver disease and Cu in cholestasis may be attenuated by Zn therapy (Figure 14). While Zn deficiency is detrimental to these patients, over dosage with Zn can result in hemolytic anemia and systemic toxicity [81,92].

Effect of zinc on iron absorption: Zinc and Fe interact competitively during intestinal absorption. When both nutrients are ingested simultaneously in aqueous solutions at levels commonly used in supplements, there is evidence that an excess of Fe inhibits Zn absorption and that excess Zn inhibits Fe uptake. However, Fe supplementation did not affect measures of Zn status, but Zn supplementation appeared to further reduce Fe status [93].

The body maintains Fe homeostasis principally by regulating Fe absorption relative to liver Fe stores. Zn supplementation increased liver HAMP expression, leading to speculate that Zn supplementation affects HAMP translation or secretion into circulation. In contrast to the systemic regulation of Fpn1 via HAMP, DMT-1 is inversely regulated through changes in enterocyte Fe levels by affecting DMT-1 mRNA levels, suggesting specific effect of Zn supplementation on intestinal Fe transporters [94,95].

4.55	DRI (mg/day)		
Age	Male	Female	
0 through 6 months	2	2	
7 through 12 months	3	3	
1 through 3 years	3	3	
4 through 8 years	5	5	
9 through 13 years	8	8	
14 through 18 years	11	9	
19 through 50 years	11	8	
>51 years	11	8	
Pregnancy			
<18 years		12	
19 through 50 years		11	
Lactation			
<18 years		13	
19 through 50 years		12	

Table 6: Dietary reference intake of zinc (Trumbo et al. [44]).

Zinc and copper interaction: Excessive Zn supply has been shown to inhibit intestinal absorption, hepatic accumulation and placental transfer of Cu, as well as to induce clinical and biochemical signs of Cu deficiency. Excessive Cu supply can affect hepatic Zn metabolism in some species but there is no consistent evidence that Zn absorption is seriously affected. Ceruloplasmin binds only Cu whereas  $\alpha$ -2 macroglobulin binds only Zn. Both metals bind to albumin, which is involved in their transport from the intestine to the liver but at different sites on the protein. Small amounts of plasma Cu and Zn may be bound to MT, and there is evidence that Cu displace Zn from the protein *in vivo*. However, Zn supplementation offers a means of treating conditions associated with excessive Cu accumulation, whether caused by dietary intake or genetic disturbances in Cu metabolism. Thus, Zn supplements are now used to treat WD in humans [96].

Zinc deficiency and its inherited disorders: Dietary factors that reduce the availability of Zn are the most common cause of Zn deficiency; inherited defects can also result in Zn deficiency (Table 7). Both nutritional and inherited Zn deficiency produces similar symptoms. The defect of intestinal absorption of zinc in acrodermatitis enteropathica (AE) has no homologue yet in the mouse. However, the lethal milk (lm) mutant in the mouse may be homologous to a condition of zinc deficiency described in a few breastfed, low birth weight infants [19].

Acrodermatitis enteropathica (AE): AE is the most commonly described condition of the inherited forms of Zn deficiency. The symptoms of this condition include skin lesions, alopecia, diarrhea, neuropsychological disturbances, reduce immune function and death of the patient in the absence of treatment. AE was first identified as a Zn deficiency disease when it was discovered that the symptoms could be abolished by oral Zn supplementation [97].

The gene responsible for AE was recently mapped to chromosome region 8q24.3, which found on ZIP4 molecule. In conditions of zinc deficiency, ZIP4 protein was concentrated on the plasma membrane of the cells; whereas in Zn repletion cells ZIP4 was endocytosed and was mainly found in intracellular [98].

The lethal milk mouse: Lethal milk is an inherited disorder of Zn deficiency occurring in mice. Newborn mice with the "lethal milk" (*lm*) mutation develop Zn deficiency and die within a week. Lethal milk is a recessive phenotype in mice caused by a mutation on chromosome 2. In the lethal milk mouse, a defect in the secretion of Zn from the mammary gland was demonstrated where the Zn concentration in the milk was reduced by 34% relative to the normal [99].

Zinc deficiency in premature breast fed infants: An inherited form of Zn deficiency similar to that of the lethal milk mouse is found in humans. The disorder manifests itself in premature breast fed infants, who demonstrate symptoms characteristic to nutritional Zn deficiency including dermatitis, diarrhea, alopecia, loss of appetite, impaired immune function and neuropsychiatric changes. This condition has been reported in preterm babies (27 to 33 weeks gestation); and less commonly in term babies [86,100].

## Chronic liver diseases in children

Chronic liver diseases (CLDs) are defined as a persistent inflammatory condition of the liver in which the biochemical and histopathological abnormalities are present over a long period of time. It encompasses a large number of conditions having different etiologies and existing on a continuum between hepatitis and cirrhosis. Patients



Figure 14: Beneficial attributes of zinc in patients with chronic hepatobiliary diseases (Center [92]).

Zinc deficiency disorder	Genotype	Phenotype
Acrodermatitis enteropathica	Mutation in <i>ZIP4</i> gene	<ul> <li>Normal Zn level in breast milk</li> <li>Symptoms develop after weaning</li> <li>Long life supplementation is required</li> <li>Defect of zinc absorption in gut</li> </ul>
Lethal milk mouse	Mutation in <i>ZnT4</i> gene	<ul> <li>Low Zn level in breast milk</li> <li>Symptoms develop during breast feeding</li> <li>Zn supplementation is not required after weaning</li> <li>Maternal Zn supplementation effective</li> <li>Defect of Zn secretion in breast</li> <li>Zn deficiency symptoms re-appear in old age</li> </ul>
Zinc deficiency in premature breast fed infants	Unknown	<ul> <li>Low Zn level in breast milk</li> <li>Symptoms develop during breast feeding</li> <li>Zn supplementation is not required after weaning</li> <li>Maternal Zn supplementation not effective</li> <li>Defect of Zn secretion in breast</li> <li>Reoccurrence of Zn deficiency not recorded</li> </ul>

 Table 7: Genotype and phenotype of inherted zinc deficiency disorders (Ackland and Michalczyk [100]).

with CLDs may suffer from specific complications of cirrhosis such as hepatic encephalopathy, ascites and variceal bleeding [101].

In cirrhotic patients, histologically the liver shows scarring and regeneration nodules devoid of central veins, surrounded by bands of fibrous tissues which distorts the lobular architecture. The progressive scarring of the tissue in the liver leads to altered hepatic flow and increased resistance to portal blood flow, causing portal hypertension and loss of hepatic function. Patients with cirrhosis may be either in a compensated or decompensated state. In the compensated state, the patient is asymptomatic with physical findings of enlarged liver, spleen, or both; whereas in a decompensated state, patients present with symptoms of hepatic dysfunction, portal hypertension, or both [102].

**Etiology of chronic liver diseases:** There is a wide spectrum of etiologies of CLDs in children; some of them may lead to cirrhosis if left without treatment. The possible etiologies are listed in Table 8 [103].

Despite the chronicity of the underlying disease process, the duration of signs and symptoms at diagnosis may be short, and the clinical presentation may resemble an acute hepatitic illness. So, the apparent duration of disease at presentation is of little or no importance, and strict adherence to an arbitrary time requirement for the establishment of chronicity, however, diminishes the diagnostic value of clinical, biochemical, immunological, and histological findings. Therefore, whereas previously a six month duration of disease was required for a diagnosis of chronic hepatitis to be made, this is no longer mandatory, as the onset of illiness is often uncertain, which may needlessly delay therapy [104].

Chronic liver diseases can be represented as hepatocellular and cholestatic CLDs as the most common etiologies. There are many types of hepatocellular diseases ranging from infections mainly viral, autoimmune, metabolic liver diseases to cancers. The majority of liver problems are transient or acute in nature because the liver has great healing power. However, a significant percent progress to a chronic disease, of these important categories is the autoimmune hepatitis, chronic viral hepatitis and WD [105].

Cholestasis is any condition in which the flow of bile from the liver is slowed or blocked. This process occurs as a result of impaired bile formation by the hepatocyte or from obstruction to the flow of bile through the intrahepatic and extrahepatic biliary tree. Causes of neonatal and infantile cholestasis can be summarized in Table 9 [106,107].

Alagille syndrome (AGS): Alagille syndrome is an autosomic dominant embryofoetopathy, due to mutations in the gene *JAG1*. The *JAG1* gene is located on the short (p) arm of chromosome 20 between positions 12.1 and 11.23. Neonatal cholestasis is a main feature, due to the paucity of intrahepatic bile ducts. It can rarely develop into cirrhosis, but be responsible for a disabling pruritus and xanthomas. The other features are a peculiar facies, cardiac abnormalities, butterfly vertebrae, and ocular embryotoxon. The prognosis depends on the severity of the liver and heart diseases. Hepatocarcinoma has been reported [108].

**Progressive familial intrahepatic cholestasis (PFIC):** This disease is an autosomal recessive syndrome due to the defect in a hepatocanalicular transporter which is essential for the proper secretion and formation of the bile. PFIC types 1, 2 and 3 are due to mutations in the *ATP8B1 (adenosine triphosphatase, type 8B, member 1), ABCB11 (adenosine triphosphate binding cassette, subfamily B, member 11)* and *ABCB4 (adenosine triphosphate binding cassette, subfamily B, member 4)* genes, respectively. They share the potential to cause hepatocellular cholestasis, which may progress to cirrhosis and liver failure before adulthood. The variable clinical features are jaundice, pruritus, hepatospleenomegaly, persistent diarrhea with fat malabsorption and protein loss, leading to poor growth and short stature [109].

**Cystic fibrosis (CF):** Cystic fibrosis is a multisystem disease; mutations are caused by an alteration in a single gene on chromosome 7q31.2 is called *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene, is responsible for the disease. CFTR functions as a chloride channel and may regulate other cellular transport pathways. The lungs and pancreas are the organs classically affected by CF, but liver disease has been increasingly recognized, ranges in severity from focal biliary fibrosis to cirrhosis with portal hypertension and chronic liver failure [110].

**Neglected-biliary atrasia (BA):** Biliary atrasia is a cholangiodestructive disease affecting both the intra- and extrahepatic biliary tract, which ultimately leads to cirrhosis, liver failure and death if not treated. Up to 10% of cases have other congenital anomalies, such as polysplenia, asplenia, situs inversus, absence of inferior vena cava and pre-duodenal portal vein. In the children with a failed Kasai operation, liver transplantation is the only hope [111].

Immune liver disorders			
a. Autoimmune hepatitis	b.Sclerosing cholangitis	c. Overlap syndrome	
2. Chronic viral hepatitis		·	
a. Hepatitis C virus (HCV)	b. Hepatitis B virus (HBV)	c. Hepatities D virus (HDV)	
3. Metabolic liver disorders			
a. Copper storage disorders	b. I	ron storage disease	
c. Inborn errors of carbohydrates metabolism	d. l	ysosomal storage disorders	
e. Inborn errors of amino acid metabolism	f. <b>a</b> 1 antitrypsin deficiency syndrome		
g. Non alcoholic fatty liver disease	h. N	h. Mitochondrial hepatopathies	
4. Fibrocystic liver diseases	-		
a. Congenital hepatic fibrosis	b. C	aroli disease and syndrome	
c. polycystic liver disease	d.Au dise	itosomal polycystic kidney ase	
5. Vascular disorders	-		
a. Budd-Chiari syndrome	b. V	eno-Occlusive disease	

 Table 8: Causes of chronic liver diseases in children (Hardy and Kleinman [103]).

1. Infection	
a. Viral & parasitic:	i. TORCH infections ii. HIV infection iii. Parvovirus vi. Enteric viral sepsis
b. Bacterial	i. Syphilis ii. Bacterial infection outside the liver
<ul> <li>2. Structural abnormalities (bile duct obstruction)</li> <li>a. Biliary atresia</li> <li>b. Alagille syndrome</li> <li>c. Caroli disease &amp; syndrome</li> <li>d. Inspissated bile syndrome</li> <li>e. Nonsyndromic paucity</li> <li>f. Choledochal cyst</li> <li>g. Cholelithiasis and choledocholithiasis</li> </ul>	
<ol> <li>Metabolic disorders         <ul> <li>Alpha-1-antitrypsin deficiency</li> <li>Cystic fibrosis</li> <li>Neonatal hemochromatosis</li> <li>Tyrosinemia</li> <li>Carbohydrate disorders</li> <li>Lysosomal Storage disorders</li> </ul> </li> </ol>	
<ul> <li><b>Toxic injury</b></li> <li>a. Drug-induced hepatotoxicity</li> </ul>	
5. Cholestatic syndrome	
6. Idiopathic neonatal hepatitis	

 $\label{eq:table_table_table} \begin{array}{l} \textbf{Table 9:} Causes of neonatal and infantile cholestasis (Sinha et al., Zollner and Trauner [106,107]). \end{array}$ 

**Carole's syndrome:** Caroli's syndrome is characterized by multiple segmental cystic or saccular dilatations of intrahepatic bile ducts associated with congenital hepatic fibrosis. The clinical features of this syndrome reflect both the characteristics of congenital hepatic fibrosis such as portal hypertension and that of Caroli's disease named as recurrent cholangitis and cholelithiasis. The diagnosis depends on both histology and imaging methods which can show the communication between the sacculi and the bile ducts [112].

Cytomegalovirus infection (CMV): Cytomegalovirus is recognized as the most common congenital viral infection in humans and an important cause of morbidity and mortality in immunocompromised

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hosts. In children and young adults, CMV mostly presents as an asymptomatic disease or a self-limiting mild mononucleosis like syndrome [113]. However, asymptomatic congenital CMV infection is the leading cause of non hereditary sensorineural hearing loss (SNHL). Other sequelae that may be evident only after the neonatal period can include chorioretinitis, neurodevelopmental delay with mental or motor impairment, and microcephaly. Congenital CMV infection is confirmed by detection of the virus in urine, blood, or saliva within the first 3 weeks of life by culture or polymerase chain reaction (PCR) [114].

**Toxoplasma infection:** *Toxoplasma gondii* is a zoonotic parasite resulting in human infections and one of the infectious pathogens leading to uveitis and retinochorioditis [115]. In an immunocompetent host, infection is generally chronic and asymptomatic, as the immune system keeps *T. gondii* confined to cysts and the intracellular space within the muscle and brain. Primary infection in pregnant women can be transmitted to the fetus leading to miscarriage or congenital toxoplasmosis [116]. *T. gondii*, is diagnosed mainly by serological methods that are hindered by insufficient sensitivity. When it fails, it becomes necessary to rely on either direct detection of the parasite or DNA detection by PCR [117].

**Inspisated bile syndrome (IBS):** Obstructive jaundice caused by intraluminal bile plugs, sludge or gallstones is uncommon in infancy and has been known as the IBS. IBS is defined as persistent jaundice in newborns with hemolytic anemia, with elevations of both direct and indirect bilirubin. The liver histology of these newborns was similar to that of neonatal hepatitis. Rh-incompatibility and CF are currently the most common causes. Hepatobiliary surgery is a major line of therapy in this disease [105].

Alpha-1-antitrypsin deficiency (A1ATD): Alpha-1-antitrypsin deficiency is an autosomal recessive disorder, and a result from a single gene defect has been localized to chromosome 14q32. In pediatric populations, this condition is one of the common causes of neonatal cholestasis, CLDs and liver failure. It behaves very similarly to those with untreated biliary atresia in many cases, requiring liver transplantation within a year. The diagnosis is made by alpha-1-antitrypsin phenotyping [118].

Autoimmune hepatitis (AIH): Autoimmune liver diseases are divided into AIH, primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). They play an important role in the differential diagnosis of acute and chronic liver diseases [119]. AIH is a form of chronic hepatitis with unclear causative factors and is characterized by immunological and auto-immunological manifestations [120]. AIH in children is of type 1, with antinuclear or anti-smooth muscle antibodies, more frequent in teenage girls, or of type 2, with anti-liver-kidney microsomes 1 and/or anti-liver cytosol 1 antibodies, in younger children. Other autoimmune diseases may be present in the patient or his relatives [121].

**Hepatitis C virus (HCV):** Hepatitis C virus infection emerged as a major cause of transfusion acquired non-A non-B hepatitis when it was first characterized in the 1980s. It is a major cause of CLDs and a frequent indication for liver transplantation in adult programs [122]. Children at risk for HCV infection include recipients of potentially contaminated blood products or organ transplants, and infants born to HCV infected mothers. Chronic HCV infection is usually asymptomatic in children but active hepatitis, cirrhosis and hepatocellular carcinoma can occur. The development of treatment strategies for chronic hepatitis C in children has directly evolved from clinical trials in adults [123].

Hepatitis B virus (HBV): Hepatitis B virus infection is a global

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problem and the world has 350 million carriers of chronic hepatitis B. Over 50% of these have acquired their infection vertically from their mothers (mother to child transmission, MTCT). Although, immunoprophylaxis which given at birth largely prevents MTCT. Majority (>90%) of vertically acquired infection results into chronic infection, due to induction of an immune tolerant state. Hence, management of chronic HBV during pregnancy and strategies to prevent MTCT would go a long way in global control of HBV infection and the morbidity and mortality associated with it [124].

Wilson's disease (WD): Wilson's disease is an autosomal recessive disorder of Cu metabolism resulting from the absence or dysfunction of Cu transporting P-type (ATPase) encoded on chromosome 13q14.3. This ATPase is expressed in hepatocytes where it is localized to the TGN and transports Cu into the secretory pathway for incorporation into Cp and excretion into the bile. Under physiologic circumstances, biliary excretion represents the sole mechanism for Cu excretion, and thus affected individuals have progressive Cu accumulation in the liver. When the capacity for hepatic storage is exceeded, cell death ensues with Cu release into the plasma, hemolysis, and tissue deposition. Presentation in childhood may include chronic hepatitis, asymptomatic cirrhosis, or acute liver failure. In young adults, neuropsychiatric symptoms predominate and include dystonia, tremor, personality changes, and cognitive impairments secondary to Cu accumulation in the central nervous system. The laboratory diagnosis of WD is confirmed by decreased serum Cp, increased urinary Cu content, and elevated hepatic Cu concentration [53].

**Glycogen Storage Diseases (GSD):** Inherited defects in enzymes that regulate glycogen synthesis or catabolism, primarily in the liver and/or muscle, are responsible for this group of diseases. Some enzymes defects are confined to the liver and are associated with hepatomegaly and hypoglycemia, where others affect only muscle and result in muscle cramps, weakness and myopathy. GSD is inherited as an autosomal recessive pattern; GSD IX is inherited as an X-linked recessive pattern [125,126].

**Crigler-Najjar syndrome (CN):** Crigler-Najjar syndrome is the result of defective uridine diphosphate glucuronosyl transferase activity due to mutations in the gene *UDP-glucuronosyl transferase 1 family, polypeptide A1 (UGT1A1)* located on chromosome 2q37. This result in unconjugated hyperbilirubinemia, which when untreated can lead to kernicterus. This is a condition of severe neural injury associated with deep yellow staining of the basal ganglia, cerebellum and bulbar nuclei [127].

**Budd-Chiari syndrome (BCS):** Budd-Chiari syndrome is an uncommon condition induced by thrombotic or non thrombotic obstruction to hepatic venous outflow that results in ascites and liver enlargement. It should be suspected in patients with abdominal pain, distention and splenic enlargement, particularly in association with thrombophilia. Patients may progress to cirrhosis and show the signs of liver failure. Unlike adults, children may have only firm hepatomegaly and ascites may be absent [128].

**Niemann–Pick disease type C (NPC):** Niemann-Pick Disease type C (NPC) is an autosomal recessive neurodegenerative and lysosomal storage disease that exhibits impaired intracellular cholesterol and lipid transport. It is caused by mutations in the gene *NPC1* (95%) on chromosome 18q11.2 or the gene *NPC2* (5%) on chromosome 14q24.3. The NPC phenotype is characterized by progressive neurological deterioration. Typical findings and symptoms include splenomegaly, ataxia, seizures, gelastic cataplexy, and dementia [129].

**Dubin Johnson syndrome (DJS):** Dubin-Johnson syndrome is a chronic, intermittent jaundice, mostly of conjugated hyperbilirubinemia. DJS is caused by a mutation in the *ATP-binding cassette, subfamily C, member 2 (ABCC2)* gene on chromosome 10q24. The *ABCC2* gene provides instructions for making a protein called multidrug resistance protein 2 (MRP2). This protein acts as a pump to transport substances such as bilirubin out of the liver, kidneys, intestine, or placenta so they can be excreted from the body. The level of bilirubin is not expected to be more than 20 mg/dl in this syndrome. It shows intermittent symptoms such as chronic or intermittent jaundice, abdominal pain, weakness, nausea, vomiting, anorexia and diarrhea [130].

**Congenital hepatic fibrosis (CHF):** Congenital hepatic fibrosis is a type of group of congenital disorders described as fibropolycystic disease or ductal plate malformation with a wide clinical spectrum. It is an autosomal recessive disorder that is characterized by hepatomegaly and portal hypertention with intact lobular architecture but with superimposed periportal fibrosis. Renal cystic disease is present in most patients and most closely corresponds to autosomal recessive polycystic kidney disease (PKD) [131].

**Portal vein thrombosis (PVT):** Portal vein thrombosis refers to a total or partial obstruction of the blood flow in this vein due to a thrombus formation. It is an important cause of portal hypertension in the pediatric age group with high morbidity rates due to its main complication; the upper gastrointestinal bleeding. The major complications include hypersplenism secondary to splenomegaly, growth retardation, and portal biliopathy [132].

# **Patients and Methods**

This study included 50 children with chronic liver diseases (CLDs) from the attendants of the outpatient and inpatient clinic of Pediatrics Hepatology Department, National Liver Institute, Menoufiya University, from October 2010 to February 2012. Another 50 apparently healthy children, attended the outpatient clinic at the same department, age and sex matched without liver diseases {no hepato or spleenomegaly, normal liver function tests and negative for hepatotropic viral markers (HBV, HCV)}, were enrolled as a control group. None of the participants had received mineral supplements before blood sampling. A written informed consent was signed by the parents of each child. The study was approved by the Research Ethics Committee of the National Liver Institute, Menoufiya University.

The etiological diagnosis of the CLDs group were autoimmune hepatitis (n=7), chronic hepatitis C (n=5), Alagille syndrome (n=5), cytomegalovirus hepatitis (n=4), progressive familial intrahepatic cholestasis (n=3), neglected biliary atresia (n=3), chronic hepatitis B (n=2), glycogen storage disease (n=2), Crigler Najjar syndrome (n=2), congenital hepatic fibrosis (n=2), Niemann Pick disease (n=2), inspissated bile syndrome (n=2), toxoplasma hepatitis (n=2), Caroli syndrome (n=2), portal vein thrombosis (n=2), Wilson's disease (n=1), Dubin Johnson syndrome (n=1), Budd Chiari syndrome (n=1), alpha-1-antitrypsin deficiency (n=1) and cystic fibrosis (n=1).

All studied groups were subjected to the following:

1. Full history taking.

**2.** The following laboratory investigations:

Ten ml venous blood was withdrawn into a vacutainer tube under aseptic condition. The sample was allowed to clot naturally in a test tube

then was centrifuged and the clear supernatant serum was separated. The collected serum was divided into 2 aliquots:

- 1. The first was used for the following liver function tests: ALT, AST, GGT, ALP, albumin, total protein, total and direct bilirubin.
- **2.** The second was stored frozen at -20°C until assayed for serum Cu, Cp, Fe, TIBC, ferritin and Zn.

Etiological diagnosis was based on history, clinical examination, biochemical, immunological, serological, radiological and histopathological evaluation. Data were analyzed on SPSS (statistical package for social science) program version 13 (SPSS Inc., Chicago, Illinois, USA) on an IBM compatible computer.

## Liver function tests

Alanine amino transferase (ALT), aspartate amino transferase (AST) [133,134], alkaline phosphatase (ALP) [135,136], gamma glutamyle transpeptidase (GGT) [137], total and direct bilirubin (Bil-T & Bil-D) [138], total proteins (TP) [139] and serum albumin (ALB) [140]. They were done using automated spectrophotometer (Automated Beckman Coulter Synchron CX9 ALX, Clinical system, Clinical Chemistry Analyzer, USA), Clinical Biochemistry Department, National Liver Institute, Menoufiya University.

# Serum copper (Cu)

It is based on Colorimetric test with Dibrom-PAESA. Spectrum Diagnostic Copper reagent is intended for *in vitro* quantitative, diagnostic determination of copper in human serum on both manual and automated systems [141]. It was done using semi-automatic spectrophotometer analyzer (Biosystems BTS 310, Biosystems S.A. Costa Brava 30, Barcelona, Spain), Clinical Biochemistry Department, National Liver Institute, Menoufiya University.

**Principle of the assay:** 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.

**Reagents:** R (mono-reagent) ready for use:

Acetate buffer pH 5

\* 4-(3,5-dibromo-2-pyridylazo)-N-ethyl-sulfopropylaniline, Standard (100  $\mu g/dl).$ 

# Steps:

\*

- **1.** 1 ml of reagent was dispensed into blank, standard and sample cuvettes.
- 2. 50 µl of standard was dispensed into standard cuvette.
- 3. 50 µl of sample was dispensed into sample cuvette.
- 4. Reagents were mixed and incubated for 5 minutes at 37°C.
- 5. The absorbance of the sample  $(A_s)$  and of the standard  $(A_{st})$  against the reagent blank  $(A_b)$  were measured at wave length 580 nm by Biosystems BTS 310, as follow:

$$\Delta A_s = A_s - A_b$$

 $\Delta A_{st} = A_{st} - A_{b}$ 

# Calculation

Serum Copper conc. ( $\mu$ g/dl) = ( $\Delta$  A<sub>s</sub> /  $\Delta$  A<sub>st</sub>) x 100

Serum Copper conc. ( $\mu$ mol/l) = ( $\Delta A_s / \Delta A_{st}$ ) x 15.7

## Serum ceruloplasmin (Cp)

Radial immunodiffusion (RID) plates is used for the determination of immunoglobulins and the other proteins in biological fluids. Diffu-Plates are imported from Biocientifica S.A., Argentina [142,143]. It was done in Clinical Biochemistry Department, National Liver Institute, Menoufiya University.

**Principle of the assay:** The procedure consists in an immunoprecipitation in agarose between an antigen and its homologous antibody. It is performed by incorporating one of the two immune reactants (usually antibody) uniformly throughout a layer of agarose gel, and then introducing the other reactants (usually antigen) into wells duly punched in the gel. Antigen diffuses radially out of the well into the surrounding gel-antibody mixture, and a visible ring of precipitation forms where the antigen and antibody reacted. A quantitative relationship does exist between ring diameter and antigen concentration. While the precipitate is expanding, the ratio between ring diameter and antigen concentration logarithm is approximately linear. This relationship is useful when rapid estimation is required. At reaction completion, the ratio between ring square diameter (i.e. the area of precipitate) and antigen concentration shows a linear ratio.

## Steps:

- 1. The plate was opened and let it to stay for about 5 minutes at room temperature, allowing any possible condensation to evaporate, as it should be stored in a flat surface at 2-8°C.
- 2. Wells were filled with  $5 \mu l$  of serum or control sample.
- **3.** Wet cotton was put in the plate center to avoid agarose dehydration. Close plate tightly.
- 4. Plate was allowed to stay flat at room temperature for 48 hrs.
- **5.** End point of diffusion was indicated by a sharp precipitation ring.
- **6.** The diameter was measured accurately with a suitable device. The results were evaluated using the reference table.

## Serum iron (Fe) & total iron binding capacity (TIBC)

It is based on guanidine/ferrozine method by Spectrum Diagnostic for the *in vitro* quantitative, diagnostic determination of total iron and TIBC in human serum [144,145]. It was done using semi-automatic spectrophotometer analyzer (Biosystems BTS 310, Biosystems S.A. Costa Brava 30, Barcelona, Spain), Clinical Biochemistry Department, National Liver Institute, Menoufiya University.

#### Principle of the assay:

**Serum Iron (Fe):** Ferric ion  $(Fe^{3+})$  is released from transferrin by guanidine hydrochloride and reduced to ferrous ion  $(Fe^{2+})$  by hydroxylamine. Fe<sup>2+</sup> is reacted with ferrozine forming a colored complex. The color intensity is directly proportional to the Fe concentration. To prevent Cu interference, cupric ions are bound to thiourea.

Transferrin-Fe<sup>3+</sup> 
$$\xrightarrow{\text{Guanidine-HCL}}$$
 Apotransferrin + Fe<sup>3+</sup>  
Fe<sup>3+</sup>  $\xrightarrow{\text{Hydroxylamine}}$  Fe<sup>2+</sup>  
Fe<sup>2+</sup> + Ferrozine  $\xrightarrow{}$  colored complex

**Serum total iron binding capacity (TIBC):** The transferrin in the specimen is saturated with Fe by exposure to excess Fe<sup>3+</sup>: then

unbound Fe is removed by addition of light magnesium carbonate and centrifugation. The Fe bound to protein in the supernatant is measured by the principle applied to total Fe described above.

**Reagents:** Reagent 1: buffer (pH 4.5) [acetate buffer, guanidine hydrochloride, hydroxylamine hydrochloride, thiourea]

Reagent 2: Ferrozine

Reagent 3: Ferric chloride

Reagent 4: Magnesium carbonate powder

Standard iron (ST) (200 µg/dl)

**Procedure A- serum (Fe):** By adding 5 ml of chromogen (R2) to one bottle of buffer (R1) [9 volume of R1: 1 volume of R2]; 1 ml working solution was dispensed in both reagent blank, standard and sample cuvettes.

- 1. 200 µl distilled water was dispensed into reagent blank cuvette.
- 2. 200 µl standard is dispensed into standard cuvette.
- 3. 200  $\mu l$  sample was dispensed into sample blank and sample cuvettes.
- 4. 1 ml reagent 1 was dispensed into sample blank cuvette.
- Reagents were mixed, and incubated for 5 to 10 minutes at 20 -25°C.
- 6. The absorbance of the standard (A<sub>st</sub>) and sample (A<sub>s</sub>) were measured against reagent blank (A<sub>b</sub>), and the absorbance of sample blank (A<sub>sb</sub>) was measured against distilled water (A<sub>dw</sub>) within 30 minutes at wavelength 546 nm by Biosystems BTS 310, as follow:

$$\Delta A_{s} = A_{s} - A_{b}$$
$$\Delta A_{st} = A_{st} - A_{b}$$

$$\Delta A_{sb} = A_{sb} - A_{dv}$$

#### Calculation

Serum iron conc. ( $\mu g/dl$ ) = ( $\Delta A_s - \Delta A_{sb}$ ) /  $\Delta A_{st}$ 

#### Procedure B- serum (TIBC):

- 1. 1 ml reagent 3 and 0.5 ml test specimen were pipetted into a labeled tube.
- 2. Reagents were mixed thoroughly and let stand for 5-30 minutes at 15-25°C.
- **3.** One spoonful of reagent 4 was added to each tube, mixed thoroughly and leaved for 30 minutes at 15-25°C then mixed several times during the incubation period.
- 4. Reagents were centrifuged at 3000 r.p.m for 10 minutes.
- 5. The supernatant was removed carefully into a clean tube and the serum iron was determined as described above in procedure A within an hour.

#### Calculation:

Serum TIBC = iron in supernatant  $\times$  3 (dilution)

**Transferrin saturation (TS):** Transferrin saturation is the percent of transferrin that has iron bound to it. TS were calculated from the equation:

Transferrin saturation $(TS) =$	Serum iron (Fe)
Transferrin Saturation (15) –	Total Iron Binding Capacity (TIBC)
[13,146,147].	

## Serum ferritin

The UBI MAGIWEL<sup>\*\*</sup> FERRITIN QUANTITATIVE is a device of solid phase enzyme linked immunosorbent assay (ELISA). This test kit provides quantitative measurement of ferritin in human serum to aid in the diagnosis of diseases affecting Fe metabolism, such as HCC (Fe overload) and ID anemia [148,149]. It was done using ELISA reader apparatus (Thermo Scientifc Multiskan\* FC, Microplate Photometer, Thermo Scientific - Part of Thermo Fisher Scientifc, USA), Clinical Biochemistry Department, National Liver Institute, Menoufiya University.

**Principle of the assay:** In this test, the wells which coated with specific anti-ferritin antibodies are incubated with samples (standards and unknown samples) and other anti-ferritin antibodies conjugated with horseradish peroxidase. The amount of bound peroxidase is proportional to the concentration of the ferritin present in the sample. The unbound conjugate is washed off with water. Upon the addition of the TMB (3, 3\, 5, 5\ tetramethyl benzidine) substrate, the intensity of color development is proportional to the amount of ferritin in the samples, and is measured by using 450 nm microtiter plate readers. The ferritin concentrations of samples are obtained by reference to the standards.

The standard curve is obtained by plotting the absorbance (Y-axis) versus the corresponding concentration of standards (X-axis). The ferritin concentrations of samples, which are run concurrently with standards, can be determined from the standard curve.

## Materials provided:

- 1. Microwell Strips: Anti-Ferritin antibodies coated wells, 96 wells.
- **2.** Enzyme Conjugate (11 ml): Anti-Ferritin Ab conjugated to horse- radish peroxidase.
- **3.** Sample Diluent or Zero Standard (11 ml): Phosphate buffered saline with protein.
- **4.** Reference Standard Set (0.5 ml each): Human ferritin standards in the phosphate buffered saline with protein. Five levels of standards are calibrated to 15, 50, 200, 400 and 800 ng/ml.
- 5. LOW and HIGH Controls (0.5 ml each).
- **6.** TMB Solution (11 ml): Buffer solution containing hydrogen peroxide and TMB.
- 7. Washing Buffer Concentrate (10 ml): Prepare working washing solution by adding 10 ml washing buffer concentrate into 990 ml distilled water.
- 8. Stop Solution (11 ml): 2 N (HCL).
- 9. Well holder for securing individual well.

# Steps:

- 1. All reagents and samples were brought to room temperature and shaked gently before beginning the test.
- 2. The desired number of coated wells was secured in the holder.
- 3. 10  $\mu l$  of serum sample, control and standard were dispensed

into the assigned wells.

- 4. 100 µl of enzyme conjugate was dispensed into each well.
- 5. Reagents were incubated for 30 minutes at room temperature.
- 6. Incubation mixture were removed and rinsed the wells five times with washing buffer (300  $\mu$ l / well / each rinse).
- 100 µl of TMB solution was dispensed into each well including the blank wells.
- 8. Reagents were incubated for 15 minutes at room temperature.
- 9. 50 µl of stop solution was added to each well and read at 450 nm.

**Calculation:** The concentration (X) of Reference Standard is plotted against its absorbance (Y) on a full logarithmic graph paper. The ferritin value of patient is obtained by reference to the standard curve.

## Serum zinc (Zn)

It is based on Colorimetric test with 5-brom-PAPS. Greiner Diagnostic (Germany) reagent is intended for *in vitro* quantitative, diagnostic determination of Zn in human serum on both manual and automated systems [150]. It was done using semi-automatic spectrophotometer analyzer (Biosystems BTS 310, Biosystems S.A. Costa Brava 30, Barcelona, Spain), Clinical Biochemistry Department, National Liver Institute, Menoufiya University.

**Principle of the assay:** Zinc forms with 2-(5-Brom-2-pyridylazo)-5-(N-propyl-N-sulfopropyl-amino)-phenol a red chelate complex. The increase of absorbance can be measured and is proportional to the concentration of total Zn in the sample.

Reagents: R (mono-reagent) ready for use:

- \* 5-Br-PAPS
- \* Bicarbonate buffer pH 9.8
- \* Sodiumcitrate
- Dimethylglyoxime
- \* Detergent

Standard (200 µg/dl)

## Steps:

- 1. 1 ml of reagent was dispensed into blank, standard and sample cuvettes.
- 2. 50 µl of standard was dispensed into standard cuvette.
- 3. 50 µl of sample was dispensed into sample cuvette.
- **4.** Reagents were mixed and incubated for 10 minutes at 25°C or 5 minutes at 37°C.
- 5. The absorbance of the sample A<sub>s</sub> and of the standard A<sub>st</sub> was measured against the reagent blank A<sub>b</sub> at wave length 560 nm by Biosystems BTS 310, as follow:

$$\Delta A_{s} = A_{s} - A$$

 $\Delta \mathbf{A}_{\mathrm{st}} = \mathbf{A}_{\mathrm{st}} - \mathbf{A}_{\mathrm{b}}$ 

# Calculation

Serum zinc conc. ( $\mu$ g/dl) = ( $\Delta$  A<sub>s</sub> /  $\Delta$  A<sub>st</sub>) x 200

Serum zinc conc. ( $\mu$ mol/l) = ( $\Delta_{As} / \Delta A_{st}$ ) x 30.14

#### Statistical procedure

Descriptive results were found normally distributed and so were expressed as mean  $\pm$  SD (standard deviation) or 95% confidence interval (CI). For qualitative data, significance was tested using Chi square test. For quantitative data, significance between groups was tested using the Student's *t*- test for parametric data and by Mann-Whitney test for non-parametric data. Correlation was tested using Pearson's correlation. Results were considered significant when *P*-value was less than 0.05. Statistical analysis was carried out using SPSS (statistical package for social science) program version 13 (SPSS Inc., Chicago, Illinois, USA) on an IBM compatible computer.

#### The following tests were applied:

- 1. Chi square test was applied for comparison between two groups or more as regards qualitative data.
- **2.** Student's t- test was applied for comparison between two groups as regards parametric variables.
- **3.** Mann-Whitney test was applied as a non parametric test equivalent to student t- test. It was used for comparison between two unpaired groups when their data are not homogenous.
- **4.** Pearson's correlation test was applied for measure the strength of the linear relationship between two variables.

## Figure legends:

- Box and whiskers plot: The top and bottom of each box are the 75<sup>th</sup> and 25<sup>th</sup> centiles. The line through the box is the median and the error bars are the maximum and minimum. The horizontal bar represents the significance between the designated groups.
- 2. The scatter plot figure represents the individual values of each patient.

#### Statistical abbreviation used:

- P = P-value
- t = student t-value
- SD = standard deviation
- X<sup>2</sup> = chi-square value

r = Pearson's correlation test

> 0.05 = non statistical significant difference

<0.05 & <0.01 = statistical significant difference at 0.05 and 0.01 respectively

## Results

Results of our work were presented in the following Tables 10-21 and Figures 15-29.

- 1. Both the chronic liver diseases group and control group were matched for both age and gender with no statistically significant difference between them, (*P*>0.05) (Tables 10 and 11).
- **2.** AST, ALT, ALP, GGT, total and direct bilirubin were significantly higher in the CLDs than the control group; while albumin and total protein were significantly lower in the CLDs than the control group, (P<0.01) (Table 12).
- 3. Serum Cu, Cu/Zn ratio, Fe, ferritin, and TS were significantly

higher in the CLDs than the control group; while Zn, Cp and TIBC were significantly lower in the CLDs than the control group, (P<0.01) (Table 13).

- **4.** There was a negative correlation between serum Zn level and the following parameters: serum Cu, Fe, AST and ALT (Table 14).
- 5. There was a positive correlation between serum Cu level and the following parameters: serum Fe, AST, ALT, GGT, total and direct bilirubin (Table 15).
- **6.** There was a negative correlation between serum Cp level and transaminase enzymes: (ALT and AST), (*P*<0.01) (Table 16).
- 7. There was a positive correlation between serum Cu/Zn ratio and the following parameters: ALT, AST and direct bilirubin (Table 17).
- **8.** There was a positive correlation between serum Fe level and transaminase enzymes: (AST and ALT), (*P*<0.01) (Table 18).
- **9.** There was a negative correlation between serum TIBC level and transaminase enzymes: (AST and ALT) (Table 19).
- **10.** There was a positive correlation between serum Ferritin level and liver function tests including: AST and ALP, (*P*>0.05) (Table 20).
- 11. There was a positive correlation between TS level and transaminase enzymes: (AST and ALT), (*P*<0.01) (Table 21).

## Discussion

Chronic liver diseases encompass a wide spectrum of etiologies including infectious, metabolic, genetic, autoimmune, structural, drug induced and idiopathic diseases. Many of these diseases have similar presentations and initial laboratory findings that definitive diagnosis can be made only by specialized laboratory tests and the histological examination of the liver tissue. A number of biochemical parameters are monitored routinely for the diagnosis of hepatic diseases, including AST, ALT, ALP, GGT, total protein, serum albumin, total and direct bilirubin [3,151].

Trace elements especially those involved in the antioxidant system as Zn or having oxidative properties, such as Cu and Fe, play an important role in pathological progression of CLDs. This is because these elements may have a direct hepatotoxicity (Cu and Fe) or may be decreased as a consequence of the impaired liver function (Zn), since liver illness particularly in patients with cirrhosis and/or malnutrition modifies the metabolism of this mineral, mainly regarding its distribution in tissues and its elimination [152,153].

Results of the present study showed that both the CLDs group and control group were matched for both age and gender with no

Gender	Chronic li gr (No	ver disease oup = 50)	Control (No =	group : 50)	X² test	P- value	
	No	%	No	%			
Male	27	54	25	50	0.16	> 0.05	
Female	23	46	25	50			

This table shows that there is no statistically significant difference between both the chronic liver diseases group and control group as regards gender (P > 0.05).

 Table 10: Comparison between CLDs and control group as regards gender.

Age in years	Chronic liver disease group (No = 50)	Control group (No = 50)	Mann Whitney	P- value
Mean ± SD	5.86 ± 4.76	6.62 ± 3.54	1.69	> 0.05

This table shows that there is no statistically significant difference between both the chronic liver diseases group and control group as regards age (P > 0.05).

 Table 11: Comparison between the CLDs and control group as regards age.

Studied variables	CLD Group (No = 50) Mean ± SD	Control Group (No = 50) Mean ± SD	Mann Whitney	P- value
AST (U/I)	193.68 ± 214.64	11.16 ± 5.78	8.62	< 0.01
ALT (U/I)	154.40 ± 184.85	12.32 ± 7.22	8.56	< 0.01
ALP (U/I)	257.02 ± 166.04	36.74 ± 5.74	8.62	< 0.01
GGT (U/I)	166.06 ± 195.21	14.82 ± 4.78	8.29	< 0.01
Bil-T (mg/dl)	5.3240 ± 4.81	0.77 ± 0.14	6.99	< 0.01
Bil-D (mg/dl)	3.48 ± 3.65	0.17 ± 0.04	7.46	< 0.01
ALB (g/dl)	3.26 ± 0.69	4.85 ± 0.37	8.28*	< 0.01
TP (g/dl)	6.25 ± 0.91	7.70 ± 0.32	10.62*	< 0.01

\* t- test.

This table shows that AST, ALT, ALP, GGT, total and direct bilirubin were significantly higher in the CLDs than the control group; while albumin and total protein were significantly lower in the CLDs than the control group, (P < 0.01).

Table 12: Comparison of liver function tests between the CLDs group and control group.

Studied variables	CLD Group (No = 50) Mean ± SD	Control Group (No = 50) Mean ± SD	t- test	P- value
Cu (µg/dl)	149.08 ± 17.85	94.48 ± 10.97	21.52	< 0.01
Fe (µg/dl)	115.41 ± 17.23	98.82 ± 13.56	5.35	< 0.01
Zn (µg/dl)	68.64 ± 19.47	95.92 ± 8.77	9.03	< 0.01
Cp (mg/dl)	$20.08 \pm 4.05$	31.58 ± 2.41	17.23	< 0.01
TIBC (µg/dl)	319.02 ± 22.34	363.06 ± 23.43	9.62	< 0.01
Ferritin (ng/ml)	131.58 ± 100.52	68.12 ± 12.46	4.19*	< 0.01
Serum Cu/Zn ratio	2.67 ± 1.02	$0.99 \pm 0.08$	8.62*	< 0.01
Transferrin Saturation	36.59 ± 7.32	27.46 ± 4.84	7.36	< 0.01

#### \* Mann Whitney test.

This table shows that serum Cu, Fe, ferritin, Cu/Zn ratio and TS were significantly higher in the CLDs than the control group; while serum Zn, Cp and TIBC were significantly lower in the CLD than the control group, (P < 0.01).

Table 13: Comparison of minerals and some of its ratios between CLDs and control group.

statistically significant difference between them (P>0.05) (Tables 10 and 11).

In our study, AIH and viral hepatitis were the most common causes of CLDs of children found in 7 (14%) cases each. This result is in accordance with that reported by [2,3,154].

Transaminases enzymes (ALT and AST) which are sensitive indicators for liver injury are produced by hepatocytes. Changes in cell permeability, hepatocellular degeneration, necrosis, and inflammation can cause release of ALT and AST from hepatocytes and subsequent increase of serum values. AST is a less reliable indicator of liver diseases because it is not liver specific (AST is also present in heart, skeletal muscle, kidney, and pancreas) [155].

In the present study the transaminases enzymes levels were significantly higher in the CLDs than the control group (P<0.01) (Table 12). The results are in agreement with that of [11] who observed a significant increase in ALT and AST in patients having hepatic cirrhosis or HCC compared with control.

Bilirubin is derived from hemoglobin degradation. If liver function is impaired, as with acute hepatitis or end stage liver disease, bilirubin accumulates in the blood and causes yellowing of the skin and eyes, called jaundice (bilirubin levels of greater than 2.5 mg/dl). Bilirubin is often reported as total, indirect (the amount of unconjugated bilirubin or free bilirubin that has not been attached to a glucuronide molecule), and direct (the amount of conjugated bilirubin or bilirubin that has been chemically attached to a glucuronide in the liver and then excreted from liver cells into the bile and stored in the gallbladder or transferred to the duodenum) [156,157]. The result of this work show that bilirubin level was significantly higher in the CLDs than the control group and in majority of the patients bilirubin was conjugated (P<0.01) (Table 12). The result goes with that of [158] who reported an elevation of total and direct bilirubin in patients with chronic liver diseases compared with control.

Alkaline phosphatase (ALP) is a family of Zn metaloenzymes, with a serine at the active center. Levels are increased in hepatitis, cirrhosis and CLDs especially cholestasis. Results may not be specific because ALP consists of several isoenzymes and has a widespread extrahepatic distribution (e.g., in the placenta, small intestine, WBCs, kidneys, and particularly bone). Zn deficiency in patients with CLDs also affects results of ALP [159].

Gamma glutamyl transferase (GGT) is a membrane bound glycoprotein which catalyses the transfere of  $\gamma$  glutamyl group to other peptide or amino acid. It is found in the kidney, pancreas, prostate, intestine, bone, bile ducts and liver. Although, ALP elevate in both liver diseases and bony disorders; GGT is more specific to the liver than ALP, as it is only raised in cholestatic disorders not in bone disorders [155].

The present work shows that ALP and GGT levels were significantly higher in the CLDs than the control group (P<0.01) (Table 12). The results are in agreement with the previous studies of [158,160] as they detected an increase of ALP and GGT in patients with cholestasis.

Serum proteins: Hepatocytes synthesize most serum proteins including  $\alpha$ -,  $\beta$ -globulins, albumin, and most clotting factors (but not factor VIII which is produced by the vascular endothelium, or

	Zn		
Studied variables	r	P - value	
Cu (µg/dl)	- 0.585	< 0.01	
Fe (µg/dl)	- 0.633	< 0.01	
AST (U/I)	- 0.402	< 0.01	
ALT (U/I)	- 0.429	< 0.01	
ALP (U/I)	- 0.175	NS	
GGT (U/I)	- 0.171	NS	
Total bilirubin (mg/dl)	- 0.216	NS	
Direct bilirubin (mg/dl)	- 0.269	NS	
ALB (g/dl)	0.202	NS	
TP (g/dl)	0.108	NS	

This table shows that there was a negative correlation between serum Zn and the following parameters: serum Cu, Fe, AST and ALT (P < 0.01).

Table 14: Correlation between serum Zn and all studied variables in CLDs group (Pearson's correlation).

Studied veriables	Cu		
Studied variables	r	P - value	
Fe (µg/dl)	0.699	< 0.01	
AST (U/I)	0.581	< 0.01	
ALT (U/I)	0.533	< 0.01	
ALP (U/I)	0.218	NS	
GGT (U/I)	0.353	< 0.05	
Total bilirubin (mg/dl)	0.405	< 0.01	
Direct bilirubin (mg/dl)	0.446	< 0.01	
ALB (g/dl)	- 0.203	NS	
TP (g/dl)	- 0.098	NS	

This table shows that there was a positive correlation between serum Cu and the following parameters: GGT (P < 0.05), serum Fe, AST, ALT, total and direct bilirubin (P < 0.01).

Table 15: Correlation between serum Cu and studied variables in CLDs group (Pearson's correlation).

Studied variables	Ср		
	r	P - value	
AST (U/I)	- 0.424	< 0.01	
ALT (U/I)	- 0.402	< 0.01	
ALP (U/I)	- 0.136	NS	
GGT (U/I)	- 0.065	NS	
Total bilirubin (mg/dl)	- 0.232	NS	
Direct bilirubin (mg/dl)	- 0.258	NS	
ALB (g/dl)	- 0.026	NS	
TP (g/dl)	- 0.030	NS	

This table shows that there was a negative correlation between serum Cp and transaminase enzymes (ALT and AST) (P < 0.01).

Table 16: Correlation between serum Cp and studied variables in CLDs group (Pearson's correlation).

y-globulin which is produced by B lymphocytes). Hepatocytes also synthesize proteins that aid in the diagnosis of specific disorders;  $\alpha_1$ -Antitrypsin (may be low or absent in A1ATD), ceruloplasmin (reduced in WD), transferrin (saturated with Fe in HHC) and ferritin (greatly increased in HHC)) [161].

Serum albumin: It is the most important plasma protein synthesized by the liver and it is a useful indicator of hepatic function. It is commonly decreased in CLDs because of an increase in the volume of distribution (e.g., due to ascites) and/or a decrease in hepatic synthesis [155].

In this work, total protein and serum albumin levels were

Zinc is transported in blood plasma mostly by albumin (60–70%),  $\alpha$ -2 macroglobulin (20–30%) and transferrin (10%). The concentration of serum Zn in liver patients had lower levels compared with the healthy controls. This observation might be the result of decreased liver albumin and α-2 macroglobulin synthesis, poor dietary intake or protein restriction and increased clearance of Zn [11,16].

This study shows that there was significantly lower serum Zn level in the CLDs than the control group (P < 0.01) (Table 13). This result is in accordance with that of [16] who found that children with CLDs, whether in a compensated or decompensated state had lower Zn level compared to healthy controls. As the severity of liver disease worsened,

Studied veriables	Serum Cu/Zn ratio		
Studied variables	r	P - value	
AST (U/I)	0.516	< 0.01	
ALT (U/I)	0.532	< 0.01	
ALP (U/I)	0.139	NS	
GGT (U/I)	0.196	NS	
Total bilirubin (mg/dl)	0.240	NS	
Direct bilirubin (mg/dl)	0.289	< 0.05	
ALB (g/dl)	-0.147	NS	
TP (g/dl)	-0.050	NS	

This table shows that there was a positive correlation between serum Cu/Zn ratio and the following parameters: ALT, AST (P < 0.01) and direct bilirubin (P < 0.05).

Table 17: Correlation between serum Cu/Zn ratio and studied variables in CLDs group (Pearson's correlation).

Studied variables	Fe		
	r	P - value	
AST (U/I)	0.508	< 0.01	
ALT (U/I)	0.502	< 0.01	
ALP (U/I)	0.126	NS	
GGT (U/I)	0.114	NS	
Total bilirubin (mg/dl)	0.226	NS	
Direct bilirubin (mg/dl)	0.243	NS	
ALB (g/dl)	- 0.149	NS	
TP (g/dl)	0.047	NS	

This table shows that there was a positive correlation between serum Fe and transaminase enzymes (AST and ALT) (P < 0.01).

Table 18: Correlation between serum Fe and studied variables in CLDs group (Pearson's correlation).

Of undiand warring by the s	TIBC		
Studied variables	r	P - value	
AST (U/I)	- 0.415	< 0.01	
ALT (U/I)	- 0.362	< 0.05	
ALP (U/I)	- 0.040	NS	
GGT (U/I)	0.048	NS	
Total bilirubin (mg/dl)	0.014	NS	
Direct bilirubin (mg/dl)	0.023	NS	
ALB (g/dl)	- 0.018	NS	
TP (g/dl)	0.005	NS	

This table shows that there was a negative correlation between serum TIBC and transaminase enzymes (AST and ALT) (P < 0.01 and P < 0.05 respectively).

Table 19: Correlation between serum TIBC and studied variables in CLDs group (Pearson's correlation).

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Studied variables	Ferittin		
	r	P- value	
AST (U/I)	0.346	< 0.05	
ALT (U/I)	0.254	NS	
ALP (U/I)	0.333	< 0.05	
GGT (U/I)	0.252	NS	
Total bilirubin (mg/dl)	0.190	NS	
Direct bilirubin (mg/dl)	0.194	NS	
ALB (g/dl)	- 0.217	NS	
TP (g/dl)	- 0.296	NS	

This table shows that there was a positive correlation between serum Ferritin and liver function tests including AST and ALP (P > 0.05).

Studied veriables	Transferrin saturation		
Studied variables	r	P- value	
AST (U/I)	0.538	< 0.01	
ALT (U/I)	0.513	< 0.01	
ALP (U/I)	0.096	NS	
GGT (U/I)	0.052	NS	
Total bilirubin (mg/dl)	0.135	NS	
Direct bilirubin (mg/dl)	0.147	NS	
ALB (g/dl)	- 0.095	NS	
TP (g/dl)	0.042	NS	

This table shows that there was a positive correlation between serum TS and transaminase enzymes (AST and ALT) (P < 0.01).



Zn levels decreased. On the other hand [162], recorded similar values of serum Zn in HCV cases and control. The differences between the results of these studies may be explained by the differences in severity (stages) of liver diseases included in each study.

It is known that there is a competition with the absorption of Zn between either metallothionein (MT) and Cu in the intestine. Also, increased clearance of pancreatic or intestinal fluids in the CLDs patients, leads to lose of Zn in the stool which is the main routes of Zn excretion [163,164].

Our results show that there was a negative correlation between serum Zn level and the following parameters: serum Cu, Fe, AST and ALT, (P<0.01) (Table 14). The negative correlation between Zn and each of Cu and Fe can be explained by the rational that elements with similar properties will act competitively in the biological systems. The significant negative correlation between Zn and the biochemical parameters of liver damage (AST and ALT) can reflect the presumed protective role of Zn against progression of liver diseases. The results are in accordance with [16].

Prasad and Kucuk [91], found that Zn administration has been









**Figure 18:** Comparison of total protein, albuimin, total and direct bilirubin levels tests between CLDs and control groups.

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Figure 19: Comparison serum Zn level between CLDs and control groups.





shown to inhibit the accumulation of hepatic collagen in experimentally produced hepatic necrosis and to significantly improve neurologic signs in hepatic encephalopathy in humans. Also, [165] found that dietary Zn supplementation could improve liver regeneration by increasing the expression of genes involved in hepatic cellular proliferation. So, the effect of Zn on liver repair mechanism might be modified by dietary Zn consumption.

Zinc is a critical component in the Zn finger motif of the Zn finger transcription factors, and the release of Zn from this motif leads to a loss





Figure 23: Comparison ferritin level between CLDs and control groups.





Figure 25: Comparison ceruloplasmin level between CLDs and control groups.



of the DNA binding ability of these transcription factors. Hepatocyte nuclear factor-4 $\alpha$  (HNF-4 $\alpha$ ), a Zn finger protein, is a liver enriched transcription factor, has been reported to bind the promoters of more than 1000 genes involved in most aspects of hepatocyte function including cell proliferation [166].

Copper functions as a co-factor in various redox enzymes, while it is highly toxic when it is in excess and may results in cellular damage and even HCC [167].

In our study, the mean serum Cu level in patients with CLDs was significantly higher than that of the control group (P<0.01) (Table 13). This is in agreement with the work of [2] in a study on patients with CLDs in a rural population in Egypt.

It is known that CLDs may lead to cholestasis as a result of either a functional defect in bile formation at the level of the hepatocytes, or from impairment in bile secretion and flow at the bile ducts level, causing impaired biliary excretion of Cu and excess Cu absorption, where bile ducts are the main way to excrete Cu from the body and only small amounts appear through the urine [168].

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In the current study, there was a positive correlation between serum Cu level and the following parameters: Fe, AST, ALT, GGT, total and direct bilirubin (Table 15). This may indicate a positive correlation between serum Cu level and biochemical parameters of liver damage in children with CLDs. This result goes with that of [2].

Ceruloplasmin (Cp) is an  $\alpha$ -2 globulin that carries more than 95% of all Cu in the body. Cp is synthesized in hepatocytes as an apo-Cp and secreted into the plasma after Cu is incorporated into a metal binding motif, forming holo-Cp. Failure of Cu incorporation into Cp results in loss of its enzymatic activity and rapid degradation by plasma proteases as present in WD [169].

In the current work, the mean serum Cp level in patients with CLDs is significantly lower compared with healthy controls (P<0.01) (Table 13). This is because Cp is a protein synthesized in the liver and that the liver cells are damaged, and that goes with the work of [169,170].

In this study, there was a negative correlation between serum Cp level and transaminase enzymes: (ALT and AST) (P<0.01) (Table 16). This may indicate a negative correlation between serum Cp and biochemical parameters of liver damage in children with CLDs.

The present study shows that serum Cu/Zn ratio was significantly higher in the CLDs group than the control (P<0.01) (Table 13). The result goes with that of [11] who observed that serum Cu/Zn ratio was markedly elevated in CLDs patients than that of the control group.

In this study, there was a positive correlation between serum Cu/ Zn ratio and the following parameters: ALT, AST and direct bilirubin (Table 17). This may indicate a positive correlation between serum Cu/ Zn ratio and biochemical parameters of liver damage in children with CLDs. These results have been reported also by [11].

Excessive levels of Cu may results in a number of adverse health effects including cell death in various organs of the body as kidney and liver showing necrosis, fibrosis, abnormal biomarkers of liver injury and developmental toxicity. Many of these effects are consistent with oxidative damage to membranes or macromolecules. Cu can bind to the sulfhydryl groups of several enzymes, such as glutathione reductase, thus interfering with their protection of cells from free radicals damage [42].

Inhibitor of apoptosis proteins (IAPs) are a family of caspase inhibitors that selectively bind and inhibit caspases at both the intrinsic and extrinsic pathway. All IAPs contain 1–3 baculoviral IAP repeat (BIR) motifs. Each BIR domain is folds into a functionally independent structure that binds a Zn ion [171]. Removal of Zn from an inhibitory zinc specific enzymatic site results in a marked increase of enzyme activity and accelerating cell death. Zn is not the only metal ion influencing IAPs function. Elevated serum Cu level as in case of CLDs; results in a conformational change in IAPs, which accelerates its degradation and significantly decreases its ability to inhibit caspase family then causing cell death [172].

Elevated serum Fe level has been found in cases of hemochromatosis, hepatitis, hepatic necrosis and hemolytic anemia. Decreased levels have been associated with ID anemia, chronic blood loss, chronic disorders



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and insufficient dietary Fe [6].

The mean serum Fe level in this study was significantly higher in the CLDs group than that of the control (P<0.01) (Table 13). This is in agreement with the work of [2,11].

Also, there was a positive correlation between serum Fe level and transaminase enzymes; (AST and ALT) (P<0.01) (Table 18). This may indicate a positive correlation between serum Fe level and biochemical parameters of liver damage in children with CLDs. This is also suggested by [13].

This work shows that there was significantly lower serum TIBC level in the CLDs than the control group (P<0.01) (Table 13). This is in agreement with the work of Buyukasik et al. [13]. who found that serum TIBC decreased as liver disease progressed from hepatitis toward Class C cirrhosis.

In our study there was a negative correlation between serum TIBC level and transaminase enzymes: AST (P<0.01) and ALT (P<0.05) (Table 19). This may indicate a negative correlation between serum TIBC and biochemical parameters of liver damage in children with CLDs. This is in accordance with the previous study of [65].

The present study shows that serum ferritin level was significantly higher in the CLDs than the control group (P<0.01) (Table 13). This is in agreement with Buyukasik et al. [13] in a study on serum Fe parameters in cirrhosis and chronic hepatitis. However, the wide range of ferritin levels in the CLD group shown in Fig. 23 could be due to a history of receiving packed red blood cells which can influence ferritin levels in this irregular way in these patients.

It is reported also that acute hepatitis secondary to viral infection with hepatitis A, B, C and CMV will cause an elevation in serum ferritin indicative of the liver inflammation; but not Fe overload in this case. Also, serum ferritin concentration is a marker of varied pathophysiological events and is elevated with increased liver Fe concentration, hepatic necro-inflammation, and systemic illness, all of which may cause a deterioration in liver function and clinical status [173].

In our study there was a positive correlation between serum ferritin level and liver function tests including: (AST and ALP) (P>0.05) (Table 20). This may indicate a positive correlation between serum ferritin and biochemical parameters of liver damage in children with CLDs. This also reported by [65].

The present study shows that transferrin saturation (TS) was significantly higher in the CLDs than the control group (P<0.01) (Table 13). This is in agreement with the work of Buyukasik et al. [13] in a similar study on serum Fe parameters in adults.

In the current study there was a positive correlation between serum TS and transaminase enzymes: (AST and ALT) (P<0.01) (Table 21). This may indicate a positive correlation between TS and biochemical parameters of liver damage in children with CLDs. This goes with the work of [173].

Serum Fe was invariably increased in systemic Fe overload. Also, in many advanced cirrhosis cases, TS and serum ferritin were simultaneously elevated, which may indicate Fe overload [13,73,174].

There is a key link between Fe metabolism and pathophysiology of viral hepatitis. Increase in Fe stores (serum ferritin and TS) leads to increased response to infection of hepatitis C, resistance to interferon therapy and progression of CLDs [65,175-177]. The mechanisms by which excess Fe exerts its cytotoxic effects include reduced cellular ATP levels, impaired cellular calcium homeostasis, enhanced formation of free radicals and peroxidation of organelle membrane lipids. The exact biochemical basis for Fe toxicities is not clear, but it has been suggested that free radicals formed when free Fe is present. Free Fe can present if the amount of Fe in the liver exceeds the capacity to bind it to apo-ferritin to form holo-ferritin. Fe overload of the liver often results in fibrosis and cirrhosis [178].

Increased liver Fe concentration in humans appears to enhance the effects of other hepatotoxins such as alcohol or viruses. Lipid peroxidation can lead to structural and functional alterations in lysosomes (membrane integrity, fluidity, pH), mitochondria (hepatic mitochondrial respiration) and the endoplasmic reticulum (protein synthesis). With massive Fe overload cell death may occur. At this stage, fibrogenesis is initiated and, if the excess Fe is not removed the increased deposition of collagen progresses to cirrhosis [178].

Elements with similar physical or chemical properties will act antagonistically to each other biologically. The implication was that such metals could compete for binding sites on transporter proteins or on enzymes requiring metals as co-factors. The intestinal competition of Zn with Cu and Fe for binding to a transporter molecule (divalent metal transporter-1, DMT-1) have been regarded as a prime example of competitive biological interactions between metals with similar chemical and physical properties [96,168].

The shared absorption pathway for Zn and Fe is probably distinct for binding to DMT-1. Excess Fe inhibits Zn absorption but not Cu absorption in the small intestine. This can be explained as the DMT-1 route is not a rate limiting factor in Cu absorption *in vivo* in humans. This confirms an important role for other Cu transporters such as CTR1 in the small intestine. Where in Zn the mechanism only involves an active transport facilitated by low molecular weight ligands of pancreatic origin [85,179].

Donangelo et al. [180] found that supplementation with Fe or Zn alone at bedtime for only 6 wk improved Fe or Zn status. Fe supplementation did not affect measures of Zn status, but Zn supplementation appeared to further reduce Fe status. Also, Zn therapy may solve the problem of pathologic effects of accumulated Fe in hepatic macrophages in necro-inflammatory liver diseases [92].

When both nutrients (Fe and Zn) are ingested simultaneously in aqueous solutions at levels commonly used in supplements, there is evidence that an excess of Fe inhibits Zn absorption and that excess Zn inhibits Fe uptake. This can be explained as in blood plasma; Zn is bound to and transported by albumin (60%, low affinity) and transferrin (10%). Since transferrin also transports iron, thus excessive iron can reduce zinc absorption, and *vice versa* [93,96,181].

Zn treatment improved DMT1 and ferroprotin-1 (Fpn1) expression in enterocyte, suggesting specific effect of Zn supplementation on intestinal Fe transporters. Zn supplementation increased liver hepcidin expression, leading to speculate that Zn supplementation affects hepcidin translation or secretion into circulation [94,95].

Excess dietary Zn results in induction of intestinal Zn binding proteins (metallothionein, MT) synthesis. Because MT has a greater binding capacity for Cu than for Zn, Cu absorbed into the intestinal mucosal cells may be sequestered by MT and not absorbed systemically. Thus, Zn can protect against the pathologic accumulation of Cu in the liver, either as a consequence of an inborn error of metabolism as in WD, or as a result of cholestasis. Induction of MT in the hepatocytes

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also is believed to protect against lysosomal Cu loading and subsequent cell autolysis as Zn can consider as a protective agent in situations of oxidative stress and in situations of exposure to toxic metals [182].

Low levels of Cu in the perfusion medium resulted in an increased absorption of Zn, while medium and high Cu levels resulted in decreased Zn absorption [96]. So, Zn supplementation may solve the problem of Cu accumulation in patients of CLDs. However, nutritional Cu deficiency results in decreased expression/function of hephaestin, in turn leading to systemic Fe deficiency [183].

The fact that the metabolism of Cu and Fe are interlinked has been known long time ago. DMT-1 is a transporter responsible for intestinal Fe uptake. Electrophysiological evidence suggests that DMT-I can also be a Cu transporter [184].

Ceruloplasmin (Cp) which acts as a Cu transporter, was also shown to act as a ferrioxidase, converting  $Fe^{2+}$  into  $Fe^{3+}$ , and was capable of rapidly stimulating Fe efflux from the liver. Cp is now considered to be the critical factor in the mobilization of Fe from body stores. Hephaestin is a homologue of Cp, which is widely expressed in intestinal tissue. Fe leaves the enterocyte in the form of  $Fe^{2+}$ . In order to be bound by transferrin,  $Fe^{2+}$  must first be oxidized to  $Fe^{3+}$ , which is accomplished by the ferrioxidase activity of hephaestin [185].

As transition metals, Cu and Fe can produce the reactive oxygen species (ROS) such as hydroxyl radicals. ROS can attack DNA and cause DNA mutation, which is an element in the pathological process of liver injury. On the contrary, Zn has a stabilizing function by stabilizing the structure of DNA, RNA and ribosome. Zn reputedly protects cell membranes against lipid peroxidation, and sulphydryl groups against oxidation. It plays an important role in maintaining the conformation of proteins, functions of several transcription factors, proteins that recognize certain DNA sequences and control gene transcription. Zn protects against free radical damage and may influence immune response [168,186].

The interactions between Zn, Fe and Cu appear to be especially important. Fe supplementation for children of CLDs could then impose a potential risk for negative effects not only on growth and development but also on other functions such as immune defense through impaired Zn status. In a similar way, Zn supplementation -over dosage- may affect Cu dependent Fe metabolism and immune functions as Cu is essential for Fe transport between tissues [187].

## Summary

Chronic liver diseases involve a progressive destruction and regeneration of the liver parenchyma leading to fibrosis and cirrhosis. Chronic liver diseases are associated with a number of conditions such as viral causes, toxicity from drugs, metabolic, or autoimmune. Chronicity of liver diseases are determined either by duration of liver diseases (typically >3-6 months) or by evidence of either severe liver diseases or physical stigmata of CLDs (jaundice, clubbing, spider telangiectasia, hepatosplenomegaly and ascites).

Copper is involved in many vital processes in the body, energy production, connective tissue formation, Fe metabolism, melanin synthesis and regulation of gene expression. Excess Cu ingestion interferes with absorbtion of Zn and can lead to Zn deficiency. The classical presentation of Cu toxicity is represented by the genetic disease of Cu accumulation known as WD.

Iron is involved in a broad spectrum of crucial biologic functions including oxygen binding (hemoglobins), oxygen metabolism (oxidases, peroxidases, catalases, and hydroxylases), and electron transfer (cytochromes). Patients with CLDs have a tendency to accumulate an excessive amount of Fe in their liver parenchyma.

Zinc is an essential trace element required by all living organisms because of its critical roles both as a structural component of proteins and as a cofactor in enzyme catalysis. Zinc is present in the body almost exclusively as  $Zn^{2+}$  bound to cellular proteins. Zinc deficiency is associated with acute and chronic liver diseases. Zinc supplementation protects against toxin induced liver damage and is used as a therapy for hepatic encephalopathy.

## In conclusion, we found that

- 1- Serum Fe, Cu, ferritin, TS and Cu/Zn ratio are significantly elevated in children with CLDs.
- 2- Serum Zn, Cp and TIBC are significantly decreased in children with CLDs.
- 3- Serum Fe, Cu, ferritin, TS and Cu/Zn ratio are positively correlated with biochemical parameters of liver damage in children with CLDs.
- 4- Serum Zn, Cp and TIBC are negatively correlated with biochemical parameters of liver damage in children with CLDs.

## This study recommended that

- 1- Serum Zn, Cu and Fe could be included in the routine assessment of children with CLDs.
- 2- Zinc supplementation may be encouraged in children with CLDs as it is an antioxidant and it is negatively correlated with liver damage parameters.
- 3- Caution regarding Fe and Cu intake either dietary or medicinal; should be taken in children with CLDs.
- 4- The level of certain trace elements such as Cu, Fe, Zn and Cu/ Zn ratio may serve as biomarkers for monitoring the increased severity of liver damage in children of CLDs.
- 5- Prospective, randomized studies are recommended to evaluate whether Fe and Cu depletion and Zn supplementation may reduce liver damage, fibrosis progression, and eventually morbidity and mortality in children with CLDs.

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