Sudan University of Science and Technology

Collage of Graduate Study

Assessment Plasma Levels of Alkaline phosphatase ,Total Bilirubin and Direct Bilirubin among Sudanese Patients with COVID 19 at Khartoum State.

تقييم مستويات انزيم الفوسفاتيز القلوي (ALP) والبيليروبين الكلي والمباشر في بلازما الدم للمرضى السودانيين بفيروس كوفيد 19 بولاية الخرطوم.

A dissertation submitted in partial fulfillment for the requirement of M.Sc. degree in Medical Laboratory Science (Clinical Chemistry).

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الآية

بسم الله الرحمن الرحيم

قال تعالى

وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا ﴿85﴾

صدق الله العظيم

سورة الإسراء الآية85

Dedication

To who give my life meaning

My parent father and mother

Brothers and sisters

My husband

Acknowledgement

Firstly, and finally, we grant all thanks to almighty Allah to give us power and kneed to finalize study.

I am very thankful to my supervisor Dr. Noha El jaili for her help and guidance, advice and suggestions. I would like to express special thanks to Japura isolation center staff for supporting.

Finally, I thank all our family and close friend, which encourage, believe and help this study.

Abstract

Background: Corona virus disease is challenging in everyday lives because it has devastating effects on human health and well-being and negative impact on economy.

Aim: this study was amid to assess of plasma levels of alkaline phosphatase, total bilirubin and direct bilirubin among Sudanese patients with COVID 19 at Khartoum State.

Material and methods: this was case control study conducted during the period from February to June 2022. fifty patients with covid 19 collected as case group compared with fifty healthy individuals as control group. patients and control were from Jabra isolation center and Advance Diagnostic Laboratory respectively. Blood samples were collected from both groups and serum levels of (ALP, total bilirubin and direct bilirubin) were estimated using full automated instrument spin react 200. the procedure of biochemical test was performed according to manufactured instruction. statistical test was done using SPSS version 25.

Results: this study was showing no significant deference in mean ± SD of serum ALP in covid patient (98.6 ± 16.3) U\L versus control (83.7±14.4) U\L with *p* value (0.11). but mean ± SD of serum total and direct bilirubin were significant higher in covid patient (2.3±0.35, 0.79±0.73) U\L compared with control (0.79±0.23, 0.37±0.19) U\L with *p* value (0.00, 0.00) respectively.

There were no significant difference of serum total and direct bilirubin cross gender in case group with *p* value (0.69, 0.25) respectively. in other hand serum ALP show significant higher in male compared with female in covid patient with *p* value (0.04).

There was no association between biochemical parameter (ALP, total bilirubin and direct bilirubin) and study variable (age and duration of disease) in study group with *p* value (0.078, 0.56, 0.23), (0.80, 0.82, 0.92) respectively.

Conclusion: This study concluded that there was increased in the level of total and direct bilirubin in case compared with control group. an ALP levels was also increased in male compared with female in Sudanese patient with covid-19.

المستخلص

الخلفية: يمثل مرض فيروس كورونا تحديًا في الحياة اليومية لأنه له آثار مدمرة على صحة الإنسان ورفاهيته وتأثيره السلبي على الاقتصاد.

الهدف: تهدف هذه الدراسة لتقييم مستويات انزيم الفوسفاتيز القلوي والبيليروبين الكلي والمباشر في بلازما الدم بين مرضى كوفيد 19 السودانيين بولاية الخرطوم.

المواد والطرق: هذه الدراسة تم إجراؤها خلال الفترة من فبراير إلى يونيو 2022. تم جمع خمسين مريضًا مصابًا بفيروس كوفيد 19 كمجموعة حالة مقارنة مع خمسين متطوعًا سليمًا كمجموعة ضابطة. كان المرضى من مركز العزل جبره ومختبر التشخيص المتقدم على التوالي. تم جمع عينات الدم من كلا المجموعتين وتم تقدير مستويات إنزيمات الكبد (ALP، البيليروبين الكلي والمباشر) في الدم باستخدام جهاز الدوران التلقائي الكامل spin. تم إجراء اختبار الكيمياء الحيوية وفقًا لتعليمات التصنيع. تم إجراء اختبار إحصائي باستخدام الإصدار 25 من برنامج SPSS.

النتائج: لم تظهر هذه الدراسة أي اختلاف كبير في متوسط مستويات ALP في مرضي كوفيد (98.6 ± 16.3) مقابل مجموعة التحكم (83.7 ± 14.4) بقيمة p (0.11). لكن متوسط ± مقدار التشتت في مستويات البيليروبين الكلي والمباشر في الدم كان أعلى معنويًا في مرضي كوفيد (2.3 ± 0.35، 0.79 ± 0.73) مقارنة مع مجموعة التحكم (0.79 ± 0.23، 0.37 ± 0.19) بقيمة p (0.00، 0.00) على التوالي.

لم يكن هناك فرق معنوي في مستويات البيليروبين الكلي والمباشر عبر الجنس في مجموعة المرضي المصابين بكوفيد مع قيمة p (0.69، 0.25) على التوالي. من ناحية أخرى، أظهرت مستويات انزيم ALP أعلى معنويًا في الذكور مقارنة بالإناث في مرضي كوفيد بقيمة p (0.04).

لم يكن هناك ارتباط بين مستويات الكيمياء الحيوية (ALP، البيليروبين الكلي والمباشر) ومتغير الدراسة (العمر ومدة المرض) في مجموعة الدراسة بقيمة 0.078) p، 0.56، 0.23)، (0.80، 0.82، 0.92) على التوالي.

الخلاصة: لخصت هذه الدراسة إلى أن هناك زيادة في مستوى البيليروبين الكلي والمباشر في المصابين بفيروس كورنا مقارنة مع غير المصابين.

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List of Abbreviation

ACE: acetyl choline esterase.

ACE-2: angiotensin converting enzyme-2.

ALP: alkaline phosphatase.

ARDS: acute respiratory distress syndrome.

ATP: adenosine tri phosphate.

COVID 19: corona virus 19.

ERGIC: endoplasmic reticulum-Golgi intermediate compartment.

IFN: interferon.

IL: interleukin.

MERS: middle east respiratory syndrome.

OATP: organic anion transporter protein.

RNA: ribose nucleic acid.

RT PCR: real time polymerase chain reaction.

SARS: sever acute respiratory syndrome.

TGF: tumor growth factor.

TNF: tumor necrosis factor.

UDP GT: uridine phosphate glucuronyl transferase.

WHO: world health organization

1.Introduction, Rational and Objectives

1.1 Introduction:

Corona virus (covid -19) affected more than 500 million confirmed cases over six million deaths which have been reported globally by WHO weekly statistical report (Ertuglu *et al*., 2020).

Patients with older age and underlying conditions like diabetes mellites, hypertension, lung disease, , however, are at increased risk for the development of severe pneumonia, resulting in the acute respiratory distress syndrome (ARDS) and multi-organ failure. (Lin *et al*.,2014) Even though the main health care focus lies in the severe pulmonary affections of the disease, it has become evident that COVID-19 can affect multiple organ systems including the liver. Being still a matter of debate, the etiology of the liver injury in these patients may be multifactorial either directly as the ACE2 receptor is expressed in cholangiocytes or indirectly as a result of a severe inflammatory response/sepsis, hypoxic or drug-induced liver injury. (Morgan, *et al*.,2020).

Within the spectrum of liver injury in COVID-19 patients, cholestasis was considered to be a rare event. elevations of alkaline phosphatase were reported in 6.1% (Kulkarni *et al*.,2020) Bile duct injury in patients with severe COVID-19 could be a result of hypoxia and severe RDS or potentially by direct infection of cholangiocytes. (simon., *et al* .2021); (Bishop *et al*.,2008).

Several studies demonstrated that elevations of liver enzymes are observed in up to 50% of the patients. (Huang, *et al*.,2019).

1.2 Rationale:

COVID 19 remain a common cause of mortality and morbidity and represent a major health problem with heavy social and economic aspect of life in Sudan, COVID 19 affect liver and bile duct. Studying of total bilirubin, direct bilirubin and ALP level could provide anew protocol for prevention cholangitis and bile duct disease and guide for treatment which may lead to improve prognosis of the effected individual.

1.3 Objective:

1.3.1. General objective:

To Assess plasma levels of ALP, total bilirubin and direct bilirubin among Sudanese patients with COVID 19 in Khartoum State.

1.3.2. Specific objectives:

1-To measure total bilirubin, direct bilirubin and ALP in study groups.

2-To compare between total bilirubin, direct bilirubin and ALP in case and control group.

3- To correlate between total bilirubin, direct bilirubin ALP and study variables (age and duration of disease).

2.Literature Review

2.1 Covid 19

2.1.1. Definition of covid 19

Corona viruses (CoVs) are a large family of enveloped, positive-strand RNA viruses which are globally endemic and account for a substantial fraction of upper respiratory tract infections. (Raoult *et al*., 2020).

2.1.2. Genetic Structure and Classification

Corona viruses (CoVs) are among the causative agents of human respiratory tract infections, which are enveloped, single positive-strand RNA viruses belonging to the large subfamily Coronavirinae which infect birds and mammals. The viral RNA is the largest genome known and it is between 26-32 kilo bases in length. There are seven CoVs known to cause human disease, four of them (H CoVs, namely Human CoV 229E, NL63 which is novel Covs, OC43, and HKU1; hung kung), are known as non- severe acute respiratory syndrome (SARS)-like CoVs (Raoult *et al*., 2020).

2.1.3. History and Epidemiology of Corona Virus

Corona viruses are widespread in humans and several other vertebrates and cause respiratory, enteric, hepatic, and neurologic diseases (Jiang *et al*., 2020). Over the past two decades three highly pathogenic, novel zoonotic CoVs have emerged, which cause lethal human disease, and have thus generated much media hype and public concern: SARS corona-virus (SARS-CoV now named SARS-CoV-1) discovered in November, the Middle East respiratory syndrome (MERS) corona virus (MERS-CoV) in June, 2012 and SARS-CoV-2, initially named 2019-nCoV when it was identified in December 2019 after sequencing of clinical samples from a cluster of patients with pneumonia in Wuhan, China and the disease caused by SARS-CoV-2 is named Coronavirus Diseases-2019 (COVID-19) (Raoult *et al*., 2020).

In December 31, 2019, hospitals reported a cluster of cases with pneumonia of unknown cause in Wuhan, Hubei, China, attracting great attention nationally and worldwide, On January 1, 2020, Wuhan public health authorities shut down the Huanan Seafood Wholesale Market, where wild and live animals were sold, due to a suspected link with the outbreak, On January 7, 2020, researchers rapidly isolated a novel corona virus (SARS-CoV-2, also referred to as 2019-nCoV) from confirmed infected pneumonia patients. Real-time reverse transcription polymerase chain reaction (RT-PCR) and next-generation sequencing were used to characterize it, On January 23, 2020, owing to the large flow of people during the Chinese Spring Festival, public transport was suspended in Wuhan and, eventually, in all the cities in Hubei Province to reduce the risk of further transmission. The number of RT-PCR–confirmed cases has increased rapidly. On January 30, 2020, the World Health Organization (WHO) declared COVID-19 (as it would be officially known as of February 11) to be a Public Health Emergency of International Concern and declared an epidemic. As of February 24, 2020, 80,239 cases were confirmed worldwide causing 2700 deaths. Mainland China, and especially Hubei Province, has borne the brunt of the epidemic, reporting 77,780 cases. Outside of mainland China, 33 countries have reported 2549 confirmed infections and 34 fatalities (Jiang *et al*., 2020).

2.1.4. Distribution of COVID-19 in Sudan

Sudan is the second largest country in Africa, with a total population of 43 849 260, located in the northeastern part of Africa, neighbored by countries with a high number of COVID-19 cases, such as Egypt and the Gulf Arab countries, COVID-19 has been reported in Sudan since 13 March 2020, and up to 3 July 2020 there had been 9894 confirmed cases and 616 deaths, with a 6.6% case fatality rate. The highest number of confirmed cases appeared in the Khartoum State (7214), followed by Gazira (955), Gadarif (250), Sinnar (195), North Kordufan (183), Red Sea (182) and River Nile (202), About 35% of confirmed cases were over 45 years old, and this age group showed the highest mortality

rate (6%) and the majority were in men (58%). The highest number of mortalities occurred in Khartoum (273), followed by Gazira (146), North Darfur (45), Gadarif (22) and Red Sea (38) (Altayb *et al*., 2020).

2.1.5. Transmission of Covid 19

SARS-CoV-2 is transmitted between humans through both direct (droplet and human-to-human transmission) and indirect (contaminated objects and airborne contagion) contacts from both symptomatic and asymptomatic patients (Bal *et al*., 2020). Many domestic and wild animals, including camels, cattle, cats, and bats, may serve as hosts for coronaviruses. It is considered that, generally, animal coronaviruses do not spread among humans. However, there are exceptions, such as SARS and MERS (Middle East Respiratory Syndrome), which are mainly spread though close contact with infected people via respiratory droplets from cough or sneezing. With regard to COVID-19, early patients were reported to have some link to the Huanan Seafood Market in Wuhan, China, suggesting that these early infections were due to animal-to-person transmission. However, later cases were reported among medical staff and others with no history of exposure to that market or visiting Wuhan, which was taken as an indication of human-to-human transmission (Adhikari *et al*., 2020).

2.1.6 Incubation period of COVID-19

The incubation period for COVID-19 is limited, An early analysis based on 88 confirmed cases in Chinese provinces outside Wuhan, using data on known travel to and from Wuhan to estimate the exposure interval, indicated a mean incubation period of 6.4 days with a range of 2.1 to 11.1 days and another analysis based on 158 confirmed cases outside Wuhan estimated a median incubation period of 5.0 days with a range of 2 to 14 days and these estimates are generally consistent with estimates from 10 confirmed cases in China (mean incubation period, 5.2 days) and from clinical reports of a familial cluster of COVID-19 in which symptom onset occurred 3 to 6 days after assumed exposure in Wuhan. These estimates of the incubation period of SARSCoV-2 are also in line with those of other known

human corona viruses, including SARS (mean, +5 days with range 2 to 14 days), MERS (mean, 5 to 7 days with range 2 to 14 days), and non-SARS human corona virus (mean, 3 days with range 2 to 5 days) (Lauer *et al*., 2020).

2.1.7. Viral Entry and Replication of Covid 19

The crucial step in the life cycle of any virus is an attachment and subsequent penetration into the host cell. It has been proven beyond doubt that the attachment of SARS-CoV-2 is achieved by an interaction between the spike surface glycoprotein S and angiotensin-converting enzyme 2 (ACE-2), a membrane carboxypeptidase that is ubiquitously distributed in a variety of human tissues. There are other possible receptors that are essential for the viral entry such as CD-147, glucose-regulated protein-78, the protease furin and serine protease TMPRSS2 are (Bal *et al*., 2020).

A critical proteolytic cleavage event occurred at SARS-CoV S protein at position (S2′) mediated the fusion and viral infectivity. Besides membrane fusion, the clathrin-dependent and independent endocytosis mediated SARS-CoVs entry too. After the virus enters the cells, the viral RNA genome is released into the cytoplasm and translated into two polyproteins and structural proteins, after which the viral genome begins to replicate. The newly formed envelope glycoproteins are inserted into the membrane of the endoplasmic reticulum or Golgi, and the nucleocapsid is formed by the combination of genomic RNA and nucleocapsid protein. Then, viral particles germinate into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). At last, the vesicles containing the virus particles then fuse with the plasma membrane to release the virus (Li *et al*., 2020).

2.1.8. Pathogenesis of COVID-19

Coronavirus infection is typically limited to the mucosal cells of the respiratory tract. Pneumonia caused by SARS coronavirus is characterized by diffuse edema resulting in hypoxia. The binding of the virus to angiotensin-converting enzyme-

2(ACE-2) on the surface of respiratory tract epithelium may contribute to the dysregulation of fluid balance that causes the edema in the alveolar space (Levison, 2014). The report shows that the ARDS is the main cause of death by COVID-19. One of the main mechanisms for ARDS is the cytokine storm which is a deadly uncontrolled systemic inflammatory response resulting from the release of large amounts of pro-inflammatory cytokines (IFN-α, IFN-γ, IL-1β, IL-6, IL-12, IL-18, IL-33, TNF-α, TGFβ, etc.) and chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10, etc.) by immune effector cells. This cytokine storm will trigger a violent attack by the immune system to the body, cause ARDS and multiple organ failure, and finally lead to death in severe cases (Li *et al*, 2020).

2.1.9 Complications of COVID-19

Age and sex have been shown to affect the severity of complications of COVID-19 and the rates of hospitalization and death are less than 0.1% in children but increase to 10% or more in older patients and men are more likely to develop severe complications compared to women as a consequence of SARS-CoV-2 infection and also patients with cancer and solid organ transplant recipients are at increased risk of severe COVID-19 complications because of their immunosuppressed status and the main complications reported in patients with SARSCoV-2 may include: coagulopathy, mainly disseminated intravascular coagulation, venous thromboembolism, elevated D-dimer and prolonged prothrombin time, and also include Laryngeal edema and laryngitis, Necrotizing pneumonia, cardiovascular complications, (including acute pericarditis, left ventricular dysfunction, acute myocardial injury). Also, the complication includes acute respiratory failure in approximately 5% of COVID-19, Sepsis, septic shock and multiple organ failure, higher risk of death particularly in male patients with severe disease and presence of heart injury and cardiac complications, hyperglycemia and patients receiving high doses of corticosteroids, and lastly the COVID complication include Ventilation

associated pneumonia in up to 30% of patients requiring intensive mechanical ventilation (Azer, 2020).

2.1.10 Diagnosis of covid 19

The real time reverse transcription–polymerase chain reaction (Real time RT-PCR) is one of the best and accurate laboratory methods for detecting, tracking, and studying the coronavirus. It is a method by which we can detect the presence of specific target genetic material. The real-time RT-PCR minimized the chance of false positive results because the amplification and analysis carried out in a closed system (Sethuraman *et al*., 2020). Coronaviruses have a number of molecular targets within their positive-sense, single-stranded RNA genome that can be used for PCR assays. These include genes encoding structural proteins; such as helicase (Hel), nucleocapsid (N), transmembrane (M), envelope (E) and envelope glycoproteins spike (S). There are also species-specific accessory genes that are required for viral replication, these include hemagglutinin esterase, open reading frames OR F1a and ORF1b and RNA-dependent RNA polymerase (Tang *et al*., 2020).

During infection, several types of antibodies are raised to the virus. IgM antibodies emerge first, after 5 days post-symptom onset. IgG antibodies are more tailored, and typically emerge after 10 days post-symptom onset. Many serology tests detect both IgG and IgM, which increases the specificity of the test. IgA antibodies may also increase during infection, and are typically found in mucous (Johns Hopkins, 2020).

The presence of antibodies only indicates previous SARS-CoV-2 infection. The results of serology tests can then be used to estimate the true spread of the virus through a population, even if individuals were asymptomatic or were never diagnosed (Johns Hopkins, 2020).

In real time –PCR assay the viral RNA is measured by the cycle threshold (Ct), which is defined as the number of cycles required for the fluorescent signal to cross the threshold and becomes detectable. The interpretation of result in real

time-PCR is based on Ct values for specimen; a value less than 40 is clinically reported as PCR positive. RT-PCR is highly specific but false negative result may also occur due to sampling error or inappropriate timing of sampling (Sethuraman *et al*., 2020).

2.1.11 Treatment of COVID-19

Just like SARS-CoV and MERS-CoV, there is currently no clinically proven specific antiviral agent available for SARS-CoV-2 infection. The supportive treatment, including oxygen therapy, conservation fluid management, and the use of broad-spectrum antibiotics to cover secondary bacterial infection, remains to be the most important management strategy, also rapid and early laboratory diagnosis of COVID-19 is the main focus of treatment and control (Li *et al*., 2020).

2.1.12 Prognosis of COVID-19

The elderly and patients with underlying diseases are more likely to experience a severe progression of COVID- 19. It is recommended that timely antiviral treatment should be initiated to slow the disease progression and improve the prognosis (Wu *et al*., 2020). Rapid blood tests, including platelet count, prothrombin time, D-dimer, and neutrophil to lymphocyte ratio can help clinicians to assess severity and prognosis of patients with COVID-19. The sepsis-induced coagulopathy scoring system can be used for early assessment and management of patients with critical disease (Liao *et al*., 2020).

2.1.13 Prevention of COVID-19

For the general population, at this moment there is no vaccine preventing COVID-19. The best prevention is to avoid being exposed to the virus. Airborne precautions and other protective measures have been discussed and proposed for prevention. Infection preventive and control measures that may reduce the risk of exposure include the following: use of face masks; covering coughs and sneezes with tissues that are then safely disposed of (or, if no tissues are available, use a

flexed elbow to cover the cough or sneeze); regular hand washing with soap or disinfection with hand sanitizer containing at least 60% alcohol (if soap and water are not available); avoidance of contact with infected people and maintaining an appropriate distance as much as possible; and refraining from touching eyes, nose, and mouth with unwashed hands(Adhikari *et al*.,2020).

2.2 Alkaline Phosphatases

Alkaline phosphatases are plasma membrane-bound glycoproteins. These enzymes are widely distributed in nature, including prokaryotes and higher eukaryotes, with the exception of some higher plants. Alkaline phosphatase forms a large family of dimeric enzymes, usually confined to the cell surface hydrolyzes various monophosphate esters at a high pH optimum with release of inorganic phosphate (Sadeghirishi and Yazdanparast, 2007).

Mammalian alkaline phosphatases (ALPs) are zinc containing metalloenzymes encoded by a multigene family and function as dimeric molecules. Three metal ions including two Zn^{2+} and one Mg^{2+} in the active site are essential for enzymatic activity. However, these metal ions also contribute substantially to the conformation of the ALP monomer and indirectly regulate subunit–subunit interactions (Sharma *et al*., 2012).

2.2.1 Isoforms of Alkaline Phosphatase and Their Distribution

Human ALPs can be classified into at least four tissue specific forms or isozyme as placental alkaline phosphatase (PLALP or Regan isozyme), Intestinal alkaline phosphatase (IALP), liver/bone/kidney alkaline phosphatase (L/B/K ALP), germ cell ALP (GCALP or NAGAO isozyme) (Llinas *et al*., 2006).

2.2.1.1 Placental Alkaline Phosphatase: The human placental ALP gene was mapped to chromosome 2. A homology of 87 % is found with the IAP gene. Placental ALP is a heat stable enzyme present at high levels in the placenta. A trace amount of this isoenzyme can be detected in normal sera. Part of the serum

placental-type activity originates from neutrophils. The placental ALP gene can be re-expressed by cancer cells as the Regan isoenzyme (Llinas *et al*., 2006).

2.2.1.2 Intestinal Alkaline Phosphatase: The gene encoding for intestinal ALP (IAP) is a member of the gene family mapping to the long arm of chromosome 2. IAP is partially heat-stable isozyme present at high levels in intestinal tissue. This embryonic gene can be reexpressed (in a modified form) by cancer cells and is designated as Kasahara isoenzyme (Llinas *et al*., 2006).

2.2.1.3 Germ Cell Alkaline Phosphatase: The gene encoding for germ-cell ALP (GCAP, placental like ALP) was also mapped to chromosome 2. It is heat-stable isozyme present at low levels in germ cells embryonal and some neoplastic tissues. It encodes testis/thymus ALP and can be expressed in the placenta at low levels (Llinas *et al*., 2006).

2.2.1.4 Liver/Bone/Kidney Alkaline Phosphatase: The heat-labile isozyme represents the liver/bone/kidney or tissue nonspecific (TNSALP) form. It is expressed in many tissues throughout the body and is especially abundant in hepatic, skeletal, and renal tissue (Llinas *et al*., 2006).

2.2.2 Physiological Functions of ALP

Since its first description by Suzuki and colleagues in 1907, alkaline phosphatase (ALP) has been investigated continuously and extensively. But little is known regarding the physiological function of ALPs in most tissues except that the bone isoenzyme has long been thought to have a role in normal skeletal mineralization. The natural substrates for TNSALP appear to include at least three phosphor compounds: phosphoethanolamine, inorganic pyrophosphate, and pyridoxal-50-phosphate, as evidenced by increased plasma and/or urinary levels of each in subjects with hypophosphatasia, but this is uncertain. Indeed, a variety of mechanisms have been proposed to explain the role of ALP in bone mineralization. However, apart from its role in normal bone mineralization, the

other functions of L/B/K remain obscure both in physiological and neoplastic conditions (Whyte MP, 2010; Zhu *et al*., 2012).

2.2.3 Clinical Application of ALP

Abnormal results may be due to the following conditions: Higher-than-normal ALP levels: Biliary obstruction, Bone conditions, Osteoblastic bone tumors, osteomalacia, a fracture that is healing, Liver disease or hepatitis, eating a fatty meal, if you have blood type O or B, Hyperparathyroidism, Leukemia, Lymphoma, Paget's disease, Rickets, Sarcoidosis. Lower-than-normal ALP levels: Hypophosphatasia, Malnutrition, Protein deficiency and Wilson's disease (Mehta *et al*., 2020).

2.3 Bilirubin

Bilirubin is an endogenous compound that can be toxic under certain conditions but, on the other hand, mild unconjugated hyperbilirubinemia might protect against cardiovascular diseases and tumor development. Serum bilirubin levels are often enhanced under a variety of clinical conditions (Johan Fevery, 2008).

2.3.1 Chemical Structure and formation of bilirubin

Bilirubin is formed from haem by opening of the haem ring at a carbon bridge. This cleavage is catalyzed by the enzyme haem-oxygenase, and results in liberation of iron, and in the formation of carbon monoxide and biliverdin IXa. The latter is reduced by a cytosolic enzyme biliverdin reductase to bilirubin IXa. The haem-oxygenase can temporarily be inhibited by mesoporphyrins, and this suppression results in a decreased Urinary Conjugated Bilirubin production as was shown in neonates (Drummond, Kappas, 2014).

2.3.2 Bilirubin metabolism under normal conditions

Bilirubin derives from haem present in hemoglobin and is released during breakdown of senescent erythrocytes, whereas approximately 20% of the daily production is derived from haem proteins such as the cytochrome P 450 isoenzymes, myoglobin, etc. It is formed in the monocytic macrophages of the

spleen and bone marrow and in hepatic Kupffer cells, and is released in plasma. Per 24h 3.8mg/kg or approximately 250–300mg bilirubin is formed in a normal adult (Berk *et al.*, 2009).

Because Urinary conjugated bilirubin is extremely poorly soluble in water, it is present in plasma strongly bound to albumin. Entry into the hepatocyte appears to be partly passive and partly mediated by organic anion transporter proteins (OATP 1B1 has the highest binding affinity). Bilirubin is conjugated in hepatocytic microsomes in an ester linkage with sugar moieties donated by uridine diphosphate (UDP) sugars. The conjugation is catalysed by UDP glucuroniltransferase (UDP-GT), an enzyme encoded for by the UGT1A1 gene (Bosma *et al*., 2014).

The bilirubin conjugates formed in the hepatocytes are excreted in bile against a concentration gradient and mediated by the canalicular membrane transporter multidrug resistance-related protein 2 (MRP2) also termed ABC-C2, belonging to the adenosine triphosphate (ATP)-binding cassette family. The conjugates are incorporated into mixed micelles (with bile acids, phospholipids and cholesterol) and pass with the bile into the intestine, where reductive breakdown into urobilinogen's occurs by intestinal or bacterial enzymes. A minor part undergoes deconjugation mainly by bacterial enzymes, and the ensuing UCB can undergo intestinal re-absorption, in contrast to CB (Jansen *et al*., 2012).

2.3.3 Types of bilirubin

The classification of bilirubin into direct and indirect bilirubin is based on the original Van der Bergh method of measuring bilirubin.

2.3.3.1 Total bilirubin: this is measured as the amount which reacts in 30 minutes after addition of alcohol. Normal range is 0.2-0.9 mg/dl (2-15µmol/L).

2.3.3.2 Direct Bilirubin: This is the water-soluble fraction. This is measured by the reaction with diazotized sulfanilic acid in 1 minute and Normal range 0.3mg/dl (5.1µmol/ L)

2.3.3.3 Indirect bilirubin: this fraction is calculated by difference of the total and direct bilirubin and is measure of unconjugated fraction of bilirubin (Johan Fevery, 2008).

2.3.4 Diagnostic value of bilirubin level

Bilirubin in body is a careful balance between productions and removal of the pigment. Hyperbilirubinemia is a good indicator of reduced hepatic excretory function. Serum bilirubin levels more than 17μmol/L suggest underlying liver disease. Increased unconjugated bilirubin: results from over production/ impaired uptake or conjugation of the pigment. While increased conjugated bilirubin implies impaired intra hepatic excretion of conjugated bilirubin from hepatocytes to bile ducts or bile obstruction as in surgical or obstructive jaundice (Johan Fevery, 2008).

2.4 Previous Studies

In a retrospective comparative study conducted by (Muhammad *et al*., 2020) aimed to evaluate Derangements of Liver Enzymes in Covid-19 Positive Patients of Pakistan. Out of the 77 patients, 55 were admitted in the ward, 22 were in ICU, 61 of them recovered, while 16 deaths reported. The result of study showed that the most deranged liver enzyme was found out to be and Alkaline phosphatase (14.28%). Total bilirubin was deranged in only 10 patients, however, direct bilirubin was above the normal range in 33 patients, while indirect component in only 4 patients.

In a cross-sectional study conducted by (Omrani-Nava *et al*., 2020) to evaluate the liver enzyme changes in COVID-19 patients and any possible association with prognosis. The study enrolled 93 patients with COVID-19 referring to the Mazandaran University of Medical Sciences' hospitals and 186 people from the normal population of Tabari Cohort. They found that the counts of ALP (192.6±91.2 vs. 222.2±70.6 U/L, P = 0.004) were higher in patients than in controls. The most common hepatic impairment events were increased direct

bilirubin (45.8%), ALP (17%), and total bilirubin (10.2%), in sequence. The risk of transfer to intensive and critical care units was strongly associated with elevated levels of direct bilirubin.

Another study conducted by (Abhishek Kumar *et al*., 2020) To analyses the liver function in patients with COVID-19 and their association with respect to age, sex, severity of disease and clinical features. 91 patients admitted with confirmed SARS-CoV-2 infection were included in this study and divided into asymptomatic, mild, moderate and severe groups. Liver function tests were compared among different severity groups. They found that of 91 patients with COVID-19, 70 (76.9%) had abnormal liver function. alkaline phosphatase (ALP), total bilirubin levels was 12(13.2%), 6(6.6%) cases and >2, 7(7.7%) and 2 (2.2%) cases respectively.

Also, in study by (Ram Krishan *et al*., 2020) to study COVID-19 associated variations in liver function parameters in a retrospective study. They analyzed liver function tests and inflammatory markers of170 admitted patients with confirmed COVID-19 in the tertiary care center, Post Graduate Institute of Medical Education and Research (PGIMER), India, using Roche Cobas Autoanalyzer. The study found that number of patients with normal liver enzyme levels were 63 (41.5%), while with raised levels of any of the liver enzymes were 89 (58.5%), out of which 43 (48.31%) had liver injury which manifested as increased severity in terms of intensive care unit (ICU) requirement (p=0.0005). Significantly raised levels of liver enzymes and liver injury were observed with age (p<0.0001) and in males (p=0.004). Significantly increased levels of total bilirubin (p<0.0001) were seen in patients with abnormal liver enzyme levels and liver injury as compared to patients with normal levels.

Further study by (Mcgrowder *et al*., 2021) to identify liver complications in COVID-19 patients and closely monitor the liver biochemistry tests in the management of acute hepatic injury in COVID-19 patients. A systematic search was conducted by the reviewers to identify all the relevant studies on the different

causes of liver impairment in COVID-19 patients published from 1 January 2020 to 30 April 2021. They conclude that Liver biochemistry tests such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) are deranged in COVID-19 patients with liver injury. Hepatocellular damage results in the elevation of serum AST and ALT levels in early onset disease while a cholestatic pattern that develops as the disease progress causes higher levels of ALP, GGT, direct and total bilirubin. These liver biochemistry tests are prognostic markers of disease severity and should be carefully monitored in COVID-19 patients.

3.Materials and Methods

3.1. Martials

3.1.1. Study approach:

A Quantitively method was used to measure the level of ALP, total and direct bilirubin in Covid 19 Sudanese patents during February to June 2022.

3.1.2. Study design:

This is case control study design.

3.1.3. Study population and sample size:

The study included 50 patents with Covid 19 infected from (2-14) days and 50 healthy individuals as control group.

3.1.4. Study area:

The study was conducted in Khartoum state.

3.1.5. Inclusion criteria:

Sudanese patients with Covid 19 infection and healthy individual serve as control.

3.1.6. Exclusion criteria:

Patients with hepatitis A, B, C, liver cancer, cirrhosis, ascites, jaundice, fatty liver, pancreatic disease, gall stone, biliary disease or any other liver disease, alcoholism and medication that increase ALP level were excluded.

3.1.7. Study duration:

This study was conducted during period from February 2022 to June 2022.

3.1.8 Ethical consideration:

Verbal consent was taken regarding acceptance to participate in the study and reassurance of confidentiality. Before the specimen was collected, the donors knew that this specimen was collected for research purpose.

3.2. Methods

3.2.1 Sample collection and processing:

About 3ml of venous blood were collected by safe aseptic procedure. plasma is used for the assay of ALP and total and direct bilirubin; the volume of sample is recommended that at least 2.5ml of whole blood is collected. In plasma sample; blood should centrifugated 3000 R\min for 5 minutes. After, that the plasma separated. plasma sample should be stored frozen below -20C. Sample should be thawed and mixed before assay.

3.2.2. Estimation of total bilirubin:

3.2.2.1. principle of total bilirubin:

This is an in vitro test for the quantitative determination of total bilirubin in human serum and plasma of adults and neonates on **spin react systems**.

Diazo method (special)

Total bilirubin, in the presence of a suitable solubilizing agent, is coupled with a 3.5 dichlorophenyl diazonium ion in a strongly acidic medium.

The intensity of the color of the red azo dye produced is directly proportional to the total bilirubin concentration and can be measured photometrically.

3.2.2.2. procedure of total bilirubin: appendix II.

3.2.3. Estimation of direct bilirubin:

3.2.3.1. Principle of direct bilirubin:

Acidified sodium nitrite produces nitrous acid, which reacts with sulfanilic acid (in acidic solution) to form a diazonium salt. The diazotized sulfanilic acid then reacts with bilirubin to form isomers of azobilirubin. In the direct bilirubin assay, only conjugated bilirubin is converted by the diazotized sulfanilic acid. The

intensity of the red color of azobilirubin is measured photometrically and is proportional to the direct (conjugated) bilirubin concentration.

3.2.3.2. procedure of direct bilirubin: appendix III

3.2.4. Estimation of alkaline phosphate (ALP):

3.2.4.1. Principle of ALP:

The spin react provides a colorimetric assay in accordance with a standardized method.

In the presence of magnesium and zinc ions, p-nitrophenyl phosphate is cleaved by phosphatases into phosphate and p-nitrophenol.

p-nitrophenyl phosphate + H_2O → phosphate + p-nitrophenol

The p-nitrophenol released is directly proportional to the catalytic ALP activity. It is determined by measuring the increase in absorbance.

3.2.4.2. procedure of ALP: appendix IIII

3.3. Quality control:

The precision and accuracy of all method used in this study were checked by commercially prepared control sample before it is application for measurement of the test control samples.

3.4. Data analysis:

Collected data were analyzed by using statistical package of social sciences (SPSS) T test and Pearson correlation were used.

Results

4. Results:

The present study involved 100 individuals aged range (24-82) years old divided in two group 50 patents with Covid 19 as case group and 50 healthy individuals as control group.

(Table 1) illustrate age and duration of disease, this individual age ranged between (24-108) and duration of disease (4-13 days).

(Table 2) show Comparison means level of ALP, total and direct bilirubin between case and control, result show comparison of mean ± Sd in (ALP) in case versus control the result showed no significant deference between two group (98.6±16.3), (83.7±14.4) in case compared with control with *p* value (0.11). In other hand a mean ± Sd of serum total and direct bilirubin showed significant increase in case compared with control (2.3±0.35, 0.79±0.73), (0.79±0.23, 0.37±0.19) with *p* value (0.00, 0.00) respectively.

(Table 3) show comparison of mean levels of ALP, total and direct bilirubin in case group cross gender, there is significant increase in ALP level in male compared with female in Covid patent (112±9.4, 81.6±4.5) with *p* value (0.04). but there is no significant deference between male and female infected in total and direct bilirubin (2.1±0.9, 2.2±1.1), (0.7±0.2, 0.6±0.2) with *p* value (0.69, 0.25).

(Figure 1) correlation between ALP and age in case group, the study shows that no correlation between ALP and age with *p* value (0.07) and R value (0.175).

(Figure 2) correlation between total Bilirubin and age in case group show no correlation between total bilirubin and age with *p* value (0.55) and R value (0.05).

(Figure 3) correlation between direct Bilirubin and age in case group. show no correlation between direct bilirubin and age with *p* value (0.22) R value (0.12).

Figure 4: correlation between ALP and duration of disease in case group, there was no correlation between ALP and duration of disease with *p* value (0.158) and R value (0.041).

Figure 5: correlation between total bilirubin and duration of disease in case group, there was no correlation between total bilirubin and duration of disease with *p* value (0.821) and R value (0.333).

Figure 6: correlation between direct bilirubin and duration of disease in case group, there was no correlation between direct bilirubin and duration of disease with *p* value (0.918) and R value (0.150).

Table (3.1): Descriptive Statistics of Demographic Data

Variable	N	Minimum	maximum	Mean	STD deviation
Age (year).	**100**	**24**	**82**	**61.7**	**11.5**
Duration of COVID 19 (days).	**50**	**4**	**13**	**9.2**	**2.1**

Table (3.2): Comparison means level of ALP, total and direct bilirubin between case and control.

	Case group(N=50)		Control group (N=51)		P value
Parameter	**Mean**	**Sd**	**Mean**	**Sd**	
ALP (U\L)	98.6 ± 16.3		83.7 ± 14.4		0.11
Total bilirubin	2.3 ± 0.35		0.79 ± 0.23		0.0
Direct bilirubin	0.79 ± 0.24		0.37 ± 0.19		0.0

Table (3.3): Comparison means level of ALP, total and direct bilirubin cross gender in COVID 19 group.

Parameters	Male (N= 31)		Female (N= 25)		P value
	Mean	**SD**	**Mean**	**SD**	
ALP	112.3 ± 9.4		81.6 ± 4.5		0.049
Total Bilirubin	2.09 ± 0.90		2.21 ± 1.15		0.69
Direct bilirubin	0.78 ± 0.28		0.69 ± 0.26		0.25

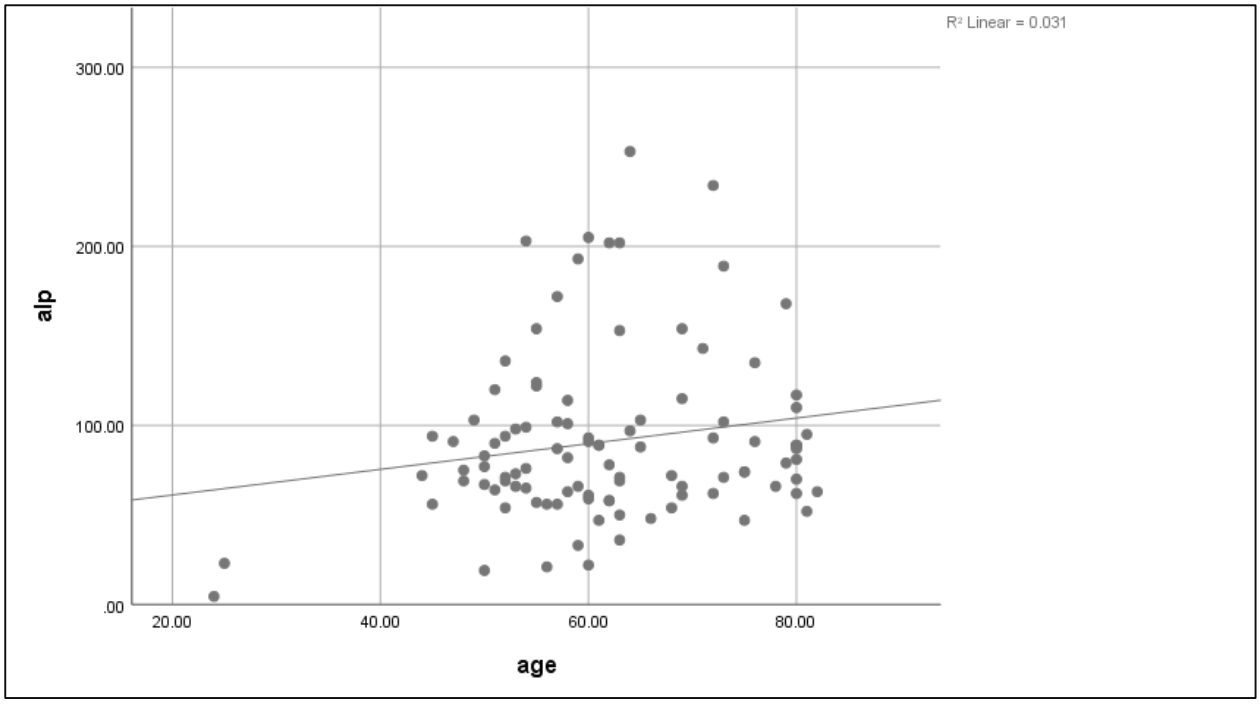

Figure 1: correlation between ALP and age in case group.

***P* value (0.07) R value (0.175).**

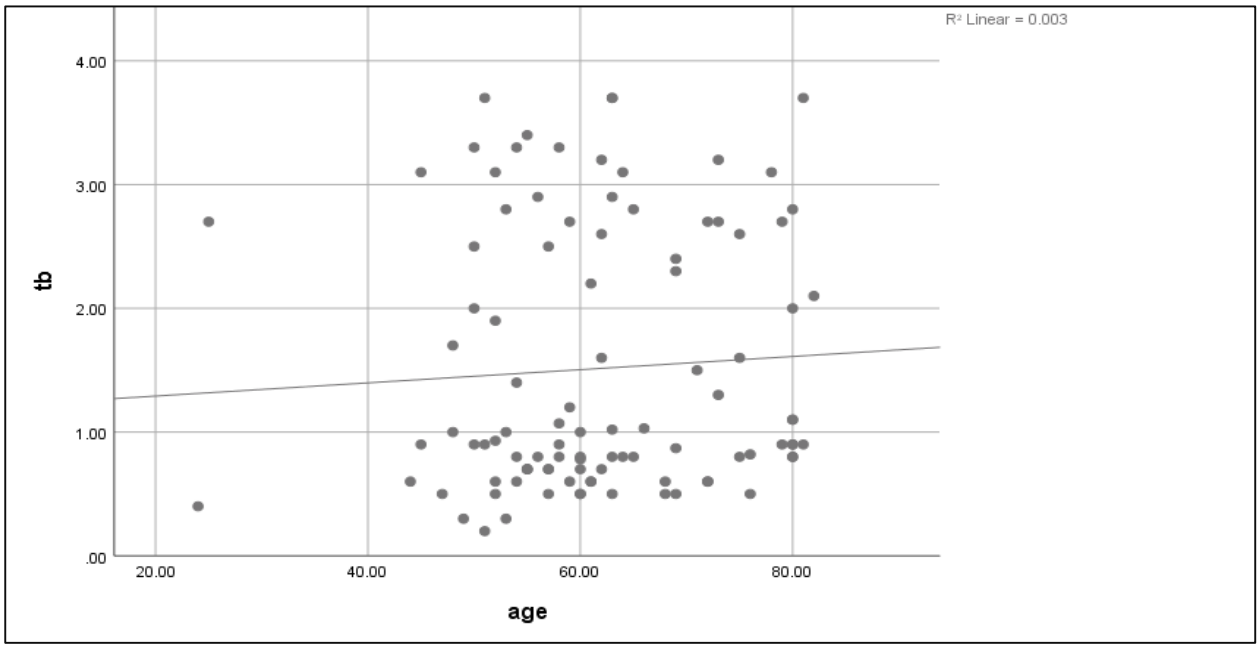

Figure 2: correlation between total Bilirubin and age in case group.

***P* value (0.55) R value (0.05).**

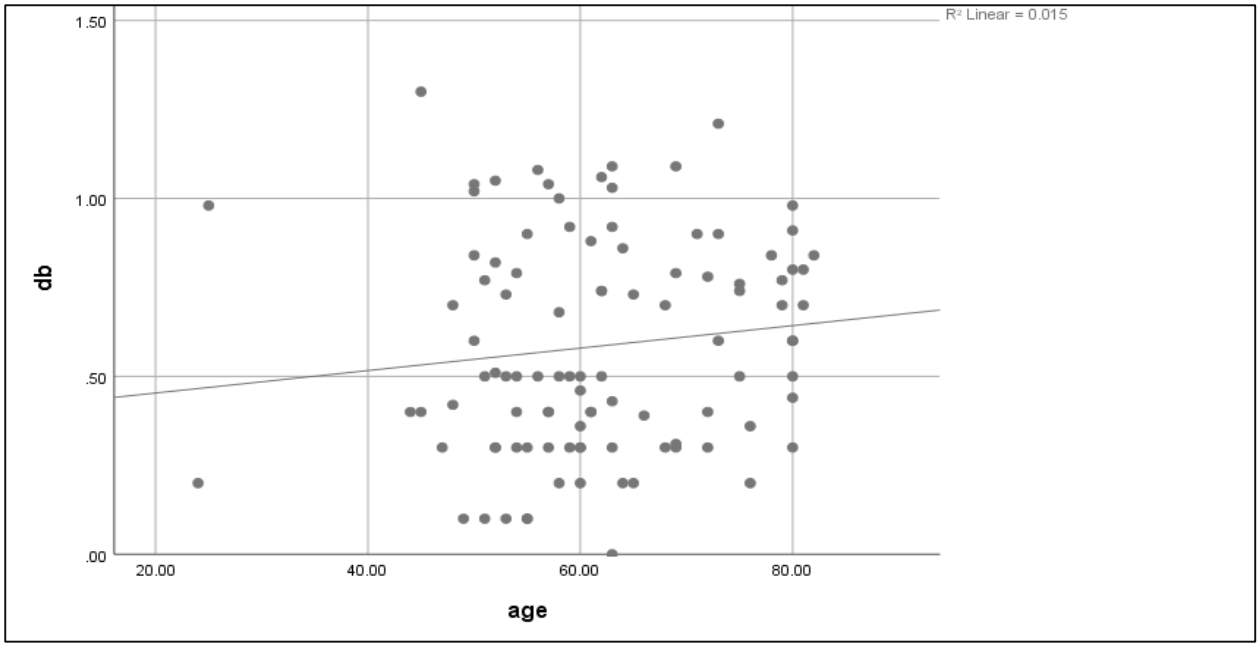

Figure 3: correlation between direct bilirubin and age in case group

***P* value (0.22) R value (0.12)**

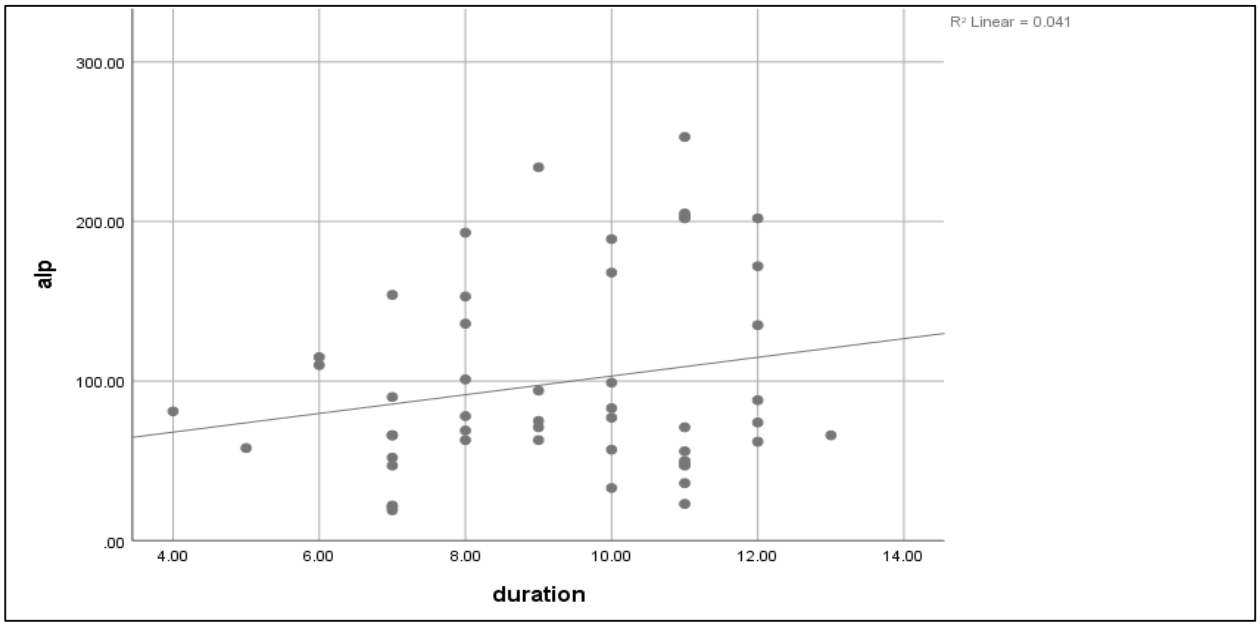

Figure 4: correlation between ALP and duration of disease in case group

***P* value (0.158) R value (0.041)**

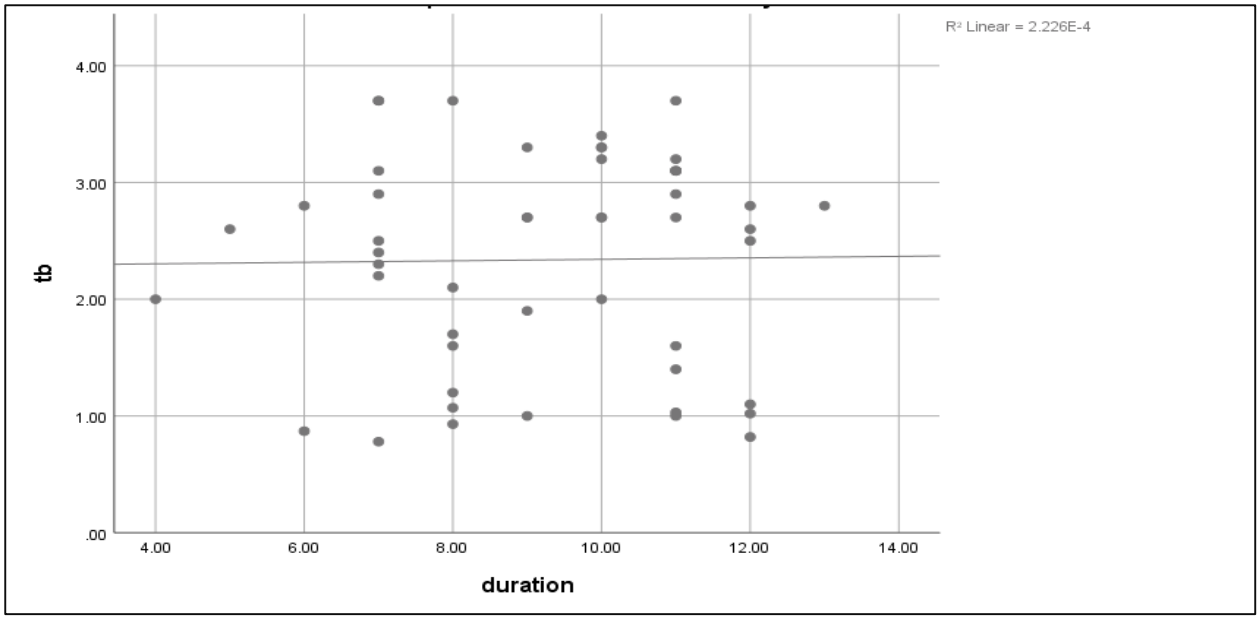

Figure 5: correlation between total bilirubin and duration of disease in case group.

***P* value (0.821) R value (0.333).**

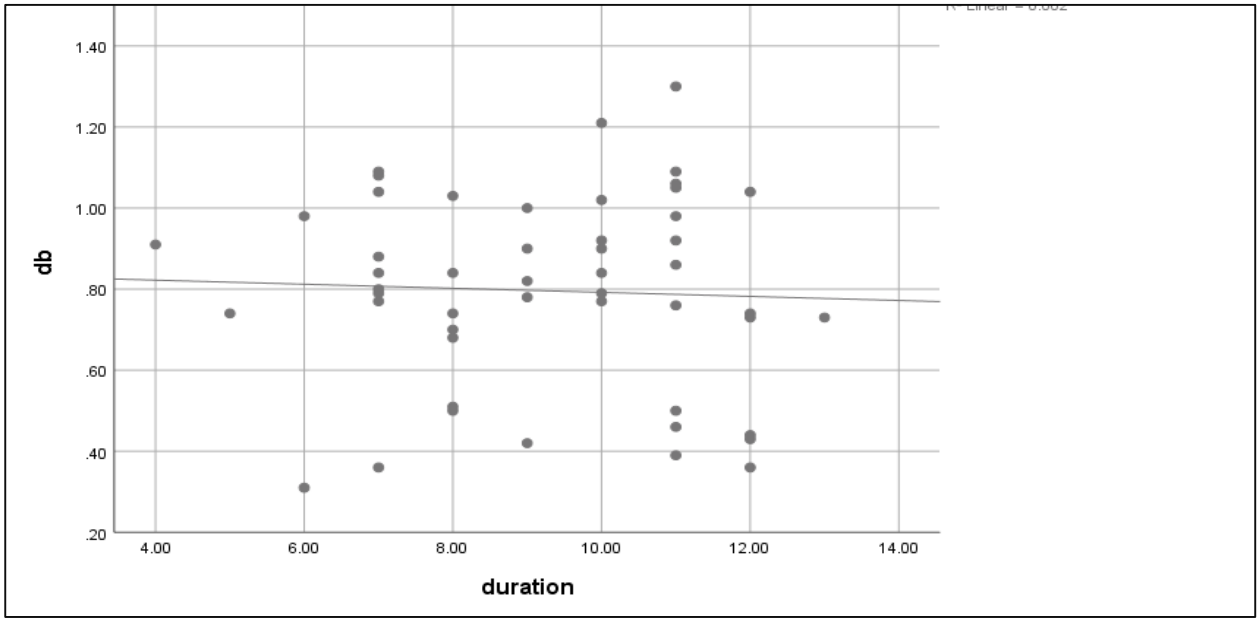

Figure 6: correlation between direct bilirubin and duration of disease in case group

***P* value (0.918) R (0.150)**

5. Discussion, Conclusion and Recommendation

5.1 Discussion

COVID-19 can affect multiple organ systems including the liver, cause liver injury and severe inflammatory. (Morgan *et al*.,2020).

The study comprises 50 hospitalize patient with Covid 19 in Japura isolation center in Sudan and 50 healthy individuals as control group, aged 24 -82 years old. The study aimed to evaluate of plasma ALP, total bilirubin and direct bilirubin among these participants.

The study showed there was insignificant different in mean of ALP in case compared to control group with *p* value (0.11). This result similar to another result carried out by (Rundk: *et al*. 2021). ACE-2 receptors is located on liver cell and is target for SARS Covs could be a reason that facilities the entry of Covs in to liver cell, moreover it has been showing that ACE bind easily to spike protein of SARS covs (Pirola and Sookoian, 2020). The other previous study demonstrate ACE is abundantly secreted by biliary epithelial cell but the abnormality is less common in ALP.

There was significant increase in mean of total and direct bilirubin in case compared to control group with *p value* = (0.00, 0.00). This result similar to another result which showed there were significant increase in mean of total and direct bilirubin (Dehange. *et al*, 2020), this may be due to ACE 2 receptor expression is enriched in cholengiocyte, so infection with SARS covs can injure cholengiocyte and cause cholestasis. Also, during the course, the patients has been treated with antipyretic agent and antiviral drugs. Including acetaminophen, oseltamivir, abidol or lopinavir, which may injure bile duct and cause cholestasis. and there may more potentially causes of bile duct obstruction, such as clotting effect SARS-covs and this need further investigation (Dehange. *et al*, 2020).

Also, this study shows there was significant increase in ALP level in male compared with female with *P* value (0.04), which is similar to another result done by (Rundk *et al*., 2021) which finding confirmed that; the higher incidence of COVID-19 in men more than women, and this could attribute to the hormonal difference. Testosterone which is known to suppress immune by increase ACE - 2 receptor expression in chlengiocyte while estrogens is known to promote immune system by suppress ACE-2 expression. (Xing *et al*.,2021). But there was no significant deference between male and female in total and direct bilirubin this result regard to another result done by Dehange. *et al*, 2020).

The study shows that no correlation between ALP, total, direct bilirubin and (age and duration of disease) with *p* value (0.070.55,0.22), (0.158, 0.821,0.918) and R value (0.176, 0.05, 0.12), (0.203, 0.033, 0.15) respectively. This result regard to (Abhishek Kumar *et al*., 2020) due low sample size.

5.2 Conclusion:

This study concluded that, there are increased in the level of total and direct bilirubin. an ALP levels also there is increased in male compared with female in Sudanese patients with COVID -19.

5.3 Recommendations

- ALP, total and direct bilirubin should be monitoring as routine investigation on covid -19 patients to avoid cholestasis and disease severity.
- Further study recommended with large sample size.
- Further studies should be conducted investigate other liver function test (Gama glutamyl transferase GGT) to asses effect of COVID upon biliary disease.

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Appendix 1

Sudan University of Science and Technology

Collage of Graduate Study

Evaluation of ALP, Total Bilirubin and Direct Bilirubin among Sudanese Patients with COVID 19 in Khartoum State.

Name……

Age……

Gender……

Male ☐ **Female** ☐

Duration of Covid19……

Chronic disease?

- **Liver cancer**
- **Cirrhosis**
- **Ascites**
- **Hepatitis**
- **Jaundice**
- **Fatty liver**
- **Pancreatic disease**
- **Gall stone**
- **Hypertension**
- **Biliary disease**
- **Autoimmune liver and bile disease**

any medication?

If yes specify ……

Investigation result:

ALP……. U\L

Total bilirubin ……...mg\dl

Direct bilirubin ……..mg\dl

Appendix II

SPINREACT

CE

BILIRUBIN T- DMSO

Bilirubin Total

DMSO. Colorimetric

Quantitative determination of bilirubin
IVD

Store at 2-8ºC

PRINCIPLE OF THE METHOD
Bilirubin is converted to colored azobilirubin by diazotized sulfanilic acid and measured photometrically. Of the two fractions presents in serum, bilirubin-glucuromide and free bilirubin loosely bound to albumin, only the former reacts directly in aqueous solution (bilirubin direct), while free bilirubin requires solubilization with dimethylsulphoxide (DMSO) to react (bilirubin indirect). In the determination of indirect bilirubin the direct is also determined, the results correspond to total bilirubin.
The intensity of the color formed is proportional to the bilirrubin concentration in the sample[1,2,3].

CLINICAL SIGNIFICANCE
Bilirubin is a breakdown product of haemoglobin.
It is transported from the spleen to the liver and excreted into bile.
Hyperbilirubinemia results from the increase of bilirubin concentrations in plasma.
Causes of hyperbilirubinemia:
Total bilirubin: Increase haemolysis, genetic errors, neonatal jaundice, ineffective erythrpoiesis, and drugs.
Direct bilirubin: Hepatic cholestasis, genetic errors, hepatocellular damage[1,6,7].
Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

REAGENTS

R 1	Sulfanilic acid	30 mmol/L
	Hydrochloric acid (HCl)	50 mmol/L
	Dimethylsulphoxide (DMSO)	7 mol/L
R 2	Sodium nitrite	29 mmol/L

PRECAUTIONS
R1: H290-May be corrosive to metals. H314-Causes severe burns and eye damage. EUH208-Contains sulphanilic acid. May produce an allergic reaction.
Follow the precautionary statements given in MSDS and label of the product.

PREPARATION
Pipette 1,5 mL of R2 into R1 content. Mix avoiding foam forming and it will be ready to use (WR). Do not use the reagent before 30 min. after the reagent preparation.

STORAGE AND STABILITY
All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8ºC, protected from light and contaminations prevented during their use. Do not use reagents over the expiration date.
Signs of reagent deterioration:
- Presence of particles and turbidity.
- Color development in R 2.

ADDITIONAL EQUIPMENT
- SPIN640 / SPIN640Plus Autoanalyser.
- General laboratory equipment.

SAMPLES
Serum or plasma, free of hemolysis[1]. Protect samples from direct light.
Stability: Bilirubin is stable at 2-8ºC for 4 days and 2 months at –20ºC.

QUALITY CONTROL
Control sera are recommended to monitor the performance of assay procedures: SPINTROL H Normal and Pathologic (Ref. 1002120 and 1002210).
If control values are found outside the defined range, check the instrument, reagents and calibrator for problems.
Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

BARCODED REAGENTS LOAD MUST BE PRECEDED OF A SPINREACT "DATABASE" COPY INTO THE ANALYZER SOFTWARE. IT IS AVAILABLE UNDER REQUEST TO SPINREACT.

SPIN640 APPLICATION

TEST INFORMATION		**REAGENT VOLUME**	
Nº	**	Vol. R1	250
Test	BILT	Vol. R2	
Full Name	Total Bilirub.	Vol. R3	
Standard nº	1	Vol. R4	
SAMPLE VOLUME		**RESULT SETUP**	
Vol. Sample Stand.	20	Decimal 0.01	Slope 1
Vol. Sample Increas.		Unit mg/dL	Inter. 0
Vol. Sample Dec			
REACTION PARAMETERS			
Reac. Type	End Point	Direction	Increase
Pri. Wave.	546	Reagent Blank	10-11
Sec. Wave.		React. Time	46-47

SPIN640Plus APPLICATION

EDIT PARAMETERS			
Test	BILT	No.	**
Full name	BILT	Print name	BILT
Reac. Type	End Point	Direction	Increase
Pri. Wave.	546	Sec. Wave.	
Unit	mg/dL	Decimal	0.01
Reagent Blank	10 - 11	React. Time	46 - 47
Vol. Sample	20 ul	R1	250 ul
Increased		R2	
Decreased		R3	
Sample blank		R4	

*The Calibration is stable until **28 days.** After this period or in case of bad results, the working reagent must be prepared again.*

REFERENCE VALUES[1]
Bilirubin Total Up to 1,10 mg/dL ≅ Up to 18,81 µmol/L

These values are for orientation purpose; each laboratory should establish its own reference range.

PERFORMANCE CHARACTERISTICS
Measuring range: From *detection limit* of 0,00526 mg/dL to *linearity limit* of 18 mg/dL.
If the results obtained were greater than linearity limit, dilute the sample 1/2 with NaCl 9 g/L and multiply the result by 2.
Precision:

	Intra-assay (n=20)		Inter-assay (n=20)	
Mean (mg/dL)	1,53	5,06	1,53	5,02
SD	0,03	0,05	0,03	0,11
CV (%)	1,73	1,01	1,92	2,18

Sensitivity: 1 mg/dL =0,05074 A.
Accuracy: Results obtained using SPINREACT reagents (y) did not show systematic differences when compared with other commercial reagents (x).
The results obtained using 50 samples were the following:
Correlation coefficient (r) [2]: 0,991.
Regression equation: y= 0,82743 x – 0,0382.
The results of the performance characteristics depend on the analyzer used.

BIBLIOGRAPHY

1. Kaplan A et al. Bilirubin. Clin Chem The C.V. Mosby Co. St Louis. Toronto. Princeton 1984; 1238-1241. 436 and 650.
2. Malloy H T. et al. The determination of bilirubin with the photoelectric colorimeter. J. Biol Chem 1937; 112, 2; 481-491.
3. Martinek R. Improved micro-method for determination of serum bilirubin. Clin Chim 1966: Acta 13: 61-170.
4. Young DS. Effects of drugs on Clinical Lab. Tests, 4th ed AACC Press, 1995.
5. Young DS. Effects of disease on Clinical Lab. Tests, 4th ed AACC 2001.
6. Burtis A et al. Tietz Textbook of Clinical Chemistry, 3rd ed AACC 1999.
7. Tietz N W et al. Clinical Guide to Laboratory Tests, 3rd ed AACC 1995.

PACKAGING
Ref: MD1001042 Cont. R 1: 5 x 40 mL
R 2: 1 x 10 mL

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Appendix III

SPINREACT

CE BILIRUBIN D- DPD

Direct Bilirubin

DPD. Colorimetric

Quantitative determination of direct bilirubin
IVD

Store at 2-8ºC

PRINCIPLE OF THE METHOD
Direct bilirubin (conjugated) couples with the diazo reagent in the presence of sulfamic acid to form azobilirubin. The intensity of color formed is proportional to the bilirubin concentration in the sample tested. The increase of absorbance at 546 nm is directly proportional to the direct bilirubin concentration.

CLINICAL SIGNIFICANCE
Bilirubin is caused by the degradation of hemoglobin and exists in two forms. Unconjugated bilirubin is transported to the liver bound by albumin where it becomes conjugated (direct) with glucuronic acid and excreted.
Hyperbilirubinemia is the result of an increase of bilirubin in plasma. Possible causes: **Total bilirubin**: Increase hemolysis, genetic, neonatal jaundice, ineffective erythropoiesis and presence of drugs. **Direct bilirubin**: Hepatic cholestasis, genetic, hepatocellular damage. Clinical diagnosis should not be made based on a single test result; it should integrate clinical and other laboratory data.

REAGENTS

R 1	Sulfamic acid	100 mM
R 2	2,4-DPD	0,5 mM
	Hydrochloric acid (HCl)	0,3 M

PRECAUTIONS
R1: H314 - Irritation or skin corrosion. / R2: H290- Corrosive to metals. H335 - May cause respiratory irritation. H314 - Irritation or skin corrosion.
R2: contains HCl and 2,4-DPD.
Follow the safety advice given in MSDS and product label.

PREPARATION
The reagents are provided in a ready to use format.

STORAGE AND STABILITY
The reagents are stable until the expiry date stated on the label when stored at
2-8ºC, protected from light and contaminations are prevented during their use. Do not use reagents over the expiration date.
Signs of reagent deterioration:
- Presence of particles and turbidity.

ADDITIONAL EQUIPMENT
- SPIN640 / SPIN640Plus Autoanalyzer.
- General laboratory equipment.

SAMPLES
Serum or plasma, free of hemolysis. Protect samples from light.
Stability of the sample: 4 days at 2-8ºC or 2 month at –20ºC.

QUALITY CONTROL
Control sera are recommended to monitor the performance of assay procedures: SPINTROL H Normal and Pathologic (Ref. 1002120 and 1002210). If control values are found outside the defined range, check the instrument, reagents and calibrator for problems.
Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

BARCODED REAGENTS LOAD MUST BE PRECEDED OF A SPINREACT "DATABASE" COPY INTO THE ANALYZER SOFTWARE. IT IS AVAILABLE UNDER REQUEST TO SPINREACT.

SPIN640 APPLICATION

TEST INFORMATION		**REAGENT VOLUME**	
Nº	**	Vol. R1	240
Test	BILI D	Vol. R2	60
Full Name	BILIRUBIN D	Vol. R3	
Standard nº	1	Vol. R4	
SAMPLE VOLUME		**RESULT SETUP**	
Vol. Sample Stand.	15	Decimal 0.01	Slope 1
Vol. Sample Increas.		Unit mg/dL	Inter. 0
Vol. Sample Dec			
REACTION PARAMETERS			
Reac. Type	End Point	Direction	Increase
Pri. Wave.	546	Reagent Blank	41-42
Sec. Wave.		React. Time	76-77

SPIN640Plus APPLICATION

EDIT PARAMETERS			
Test	BILI D	No.	**
Full name	BILI D	Print name	BILI D
Reac. Type	End Point	Direction	Increase
Pri. Wave.	546	Sec. Wave.	
Unit	mg/dL	Decimal	0.01
Reagent Blank	47 - 48	React. Time	81 - 82
Vol. Sample	15 ul	R1	240 ul
Increased		R2	60 ul
Decreased		R3	
Samole blank		R4	

*The Calibration is stable until **7 days.** After this period the Calibration must be performed again in order to obtain good results.*

REFERENCE VALUES
Direct bilirubin 0 – 0,2 mg/dL (0 – 3,42 µmol/L)
These values are for orientation purpose; each laboratory should establish its own reference range.

PERFORMANCE CHARACTERISTICS
Measuring range: From *detection limit* of 0,03 mg/dL to *linearity limit* of 9 mg/dL.
If the results obtained are greater than the linearity limit, dilute the sample 1/2 with NaCl 9 g/L and multiply the result by 2.
Precision:

	Inter assay (n= 40)		Intra assay (n= 80)	
Mean (mg/dL)	0,7458	2,444	0,7458	2,444
SD	0,05868	0,0550	0,0276	0,024
CV (%)	7,9	2,2	3,7	1,0

Sensitivity: 1 mg/dL = 0,040 Abs. units
Accuracy: Results obtained using SPINREACT reagents (y) did not show systematic differences when compared with other commercial reagents (x) on a Spintech 240 analyzer. The results obtained using 53 samples ranging from 0,06 a 9 mg/dL (1,02 to 153,9 µmol/L) were:
Correlation coefficient (r): 0,9986
Regression equation: y= 1,0056 x – 0,1046
The results of the performance characteristics depend on the analyzer used.

BIBLIOGRAPHY

1. David G Levitt and Michael D Levitt. Quantitative assessment of the multiple processes responsible for bilirubin homeostasis in health and disease. Clin Exp Gastroenterol. 2014; 7: 307–328.
2. Malloy H T. et al. The determination of bilirubin with the photoelectric colorimeter. J. Biol Chem 1937; 112, 2; 481-491.
3. Martinek R. Improved micro-method for determination of serum bilirubin. Clin Chim 1966: Acta 13: 61-170.
4. Young DS. Effects of drugs on Clinical Lab. Tests, 4th ed AACC Press, 1995.
5. Young DS. Effects of disease on Clinical Lab. Tests, 4th ed AACC 2001.
6. Burtis A et al. Tietz Textbook of Clinical Chemistry, 3rd ed AACC 1999.
7. Tietz N W et al. Clinical Guide to Laboratory Tests, 3rd ed AACC 1995.

PACKAGING

Ref: MD1001047	Cont.	R 1: 4 x 40 mL R 2: 2 x 20 mL

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Appendix IV

SPINREACT

CE

ALP-LQ

Alkaline phosphatase

p-Nitrophenylphosphate. kinetic. Liquid. DGKC

Quantitative determination of alkaline phosphatase (ALP)
IVD

Store at 2-8ºC

PRINCIPLE OF THE METHOD

Alkaline phosphatase (ALP) catalyses the hydrolysis of p-nitrophenyl phosphate at pH 10.4, liberating p-nitrophenol and phosphate, according to the following reaction:

$$\text{p-Nitrophenyl-P} + H_2O \xrightarrow{\text{ALP}} \text{p-Nitrophenol} + \text{Phosphate}$$

The rate of p-Nitrophenol formation, measured photometrically, is proportional to the catalytic concentration of alkaline phosphatase present in the sample[1,2].

CLINICAL SIGNIFICANCE

Alkaline phosphatase is an enzyme present in almost all weaves of the organism, being particularly high in bone, liver, placenta, intestine and kidney. Both increases and decreases of plasma ALP are of importance clinically.
Causes of increased plasma ALP: Paget's disease of bone, obstructive liver disease, hepatitis, hepatotoxicity caused by drugs or osteomalacia.
Causes of decreased plasma ALP: Cretinism and vitamin C deficiency[1,5,6].
Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

REAGENTS

R 1 Buffer	Diethanolamine (DEA) pH 10.4 Magnesium chloride	1 mmol/L 0,5 mmol/L
R 2 Substrate	p-Nitrophenylphosphate (*p*NPP)	10 mmol/L

PREPARATION

All the reagents are ready to use.

STORAGE AND STABILITY

All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8ºC, protected from light and contaminations prevented during their use.
Do not use reagents over the expiration date.

Signs of reagent deterioration:
- Presence of particles and turbidity.
- Blank absorbance (A) at 405 nm ≥ 1,30.

ADDITIONAL EQUIPMENT

- SPIN640 / SPIN640Plus Autoanalyzer.
- General laboratory equipment.

SAMPLES

Serum or heparinzed plasma[1]. Use unhemolyzed serum, separated from the clot as soon as possible. Stability: 3 days at 2-8ºC.

QUALITY CONTROL

Control sera and calibrators are recommended to monitor the performance of assay procedures: SPINTROL H Calibrator, SPINTROL H Normal and Pathologic (Ref. 1002011, 1002120 and 1002210).
If control values are found outside the defined range, check the instrument, reagents and technique for problems.
Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

BARCODED REAGENTS LOAD MUST BE PRECEDED OF A SPINREACT "DATABASE" COPY INTO THE ANALYZER SOFTWARE. IT IS AVAILABLE UNDER REQUEST TO SPINREACT.

SPIN640 APPLICATION

TEST INFORMATION		**REAGENT VOLUME**	
Nº	**	Vol. R1	240
Test	ALP	Vol. R2	60
Full Name	Alkaline Phosp.	Vol. R3	
Standard nº		Vol. R4	
SAMPLE VOLUME		**RESULT SETUP**	
Vol. Sample Stand.	5	Decimal 1	Slope 1
Vol. Sample Increas.		Unit U/L	Inter. 0
Vol. Sample Dec			
REACTION PARAMETERS			
Reac. Type	Kinetic	Direction	Increase
Pri. Wave.	412	Reagent Blank	0-0
Sec. Wave.		React. Time	50-70

SPIN640Plus APPLICATION

EDIT PARAMETERS			
Test	ALP	No.	**
Full name	ALK PHOSPH	Print name	ALP
Reac. Type	Kinetic	Direction	Increase
Pri. Wave.	412	Sec. Wave.	
Unit	U/L	Decimal	0
Reagent Blank	0 - 0	React. Time	57 - 77
Vol. Sample	5 ul	R1	240 ul
Increased		R2	60 ul
Decreased		R3	
Sample blank		R4	

*The Calibration is stable until **30 days**. After this period the Calibration must be performed again in order to obtain good results.*

REFERENCE VALUES[1]

Children (1-14 years) < 645 U/L

Adults 98 - 279 U/L

Factors affecting ALP activities in a normal population include exercise, periods of repaid growth in children and pregnancy.

These values are for orientation purpose; each laboratory should establish its own reference range.

PERFORMANCE CHARACTERISTICS

Measuring range: From *detection limit* of 0,6845 U/L to *linearity limit* of 1200 U/L.
If the results obtained were greater than linearity limit, dilute the sample 1/10 with NaCl 9 g/L and multiply the result by 10.

Precision:

	Intra-assay (n=20)		Inter-assay (n=20)	
Mean (U/L)	174	443	175	434
SD	0,72	1,56	6,88	11,93
CV (%)	0,41	0,35	3,93	2,75

Sensitivity: 1 U/L = 0,0003 ΔA/min.
Accuracy: Results obtained using SPINREACT reagents (y) did not show systematic differences when compared with other commercial reagents (x). The results obtained using 50 samples were the following:
Correlation coefficient $(r)^2$:0,99938.
Regression equation: y= 1,025x – 1,105.
The results of the performance characteristics depend on the analyzer used.

BIBLIOGRAPHY

1. Wenger C. et al. Alkaline phosphatase. Kaplan A et al. Clin Chem The C.V. Mosby Co. St Louis. Toronto. Princeton 1984; 1094-1098.
2. Rosalki S et al. Clin Chem 1993; 39/4: 648-652.
3. Young DS. Effects of drugs on Clinical Lab. Tests, 4th ed AACC Press, 1995.
4. Young DS. Effects of disease on Clinical Lab. Tests, 4th ed AACC 2001.
5. Burtis A et al. Tietz Textbook of Clinical Chemistry, 3rd ed AACC 1999.
6. Tietz N W et al. Clinical Guide to Laboratory Tests, 3rd ed AACC 1995.

PACKAGING

Ref: MD41233	Cont.	R 1:	4 x 40 mL
		R 2:	2 x 20 mL

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Appendix V