VARIABILITY OF BILIRUBIN VALUES IN SERUM SAMPLES WITH HIGH TRIGLYCERIDES; INTERFERENCE OR CONGENITAL LIVER SYNDROMES

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ABSTRACT
Interference between measurements of distinct blood serum components can lead to a false interpretation of the results and a delay in disease recognition. The aim of this work is to identify and interpret the variability of bilirubin measurements with respect to high values of triglycerides and variable values of cholesterol in serum samples, in order to allow for a better diagnosis of congenital liver syndromes, with a lower error rate.

Method: The study was conducted on 160 patients with 10 major blood tests performed: (a) 5 substrates/chemistries: cholesterol, total bilirubin, conjugated bilirubin, un-conjugated bilirubin, triglycerides, and (b) 5 enzymatic tests: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gama-glutamyltransferase (GGT). Additionally, hepatic viral markers (Ag HBS, Anti HCV) were analyzed, as well as hemo grams with differential count and reticulocytes. Results: The studies revealed that: (1) 90% of the patients (n=.144) showed normal blood-work; (2) in 6.8% of cases, there was an increase in the values of both total bilirubin and un-conjugated bilirubin, accompanied by normal liver enzymes, but high triglyceride values. After a 1:5 dilution and reanalysis of the samples with high triglyceride values, only 2.8% showed normal un-conjugated bilirubin values (negative predictive value=66%), while 4% showed the same high un-conjugated bilirubin values, pointing toward a congenital liver syndrome (positive predictive value=72%). Conclusions: The laboratory physician must detect the common interferences and he must make the review of potential clinical impact.


Keywords: Bilirubin; Cholesterol; Congenital; Interference; Liver; Syndrome; Triglycerides.

1. INTRODUCTION
Good communication between the laboratory staff and clinicians doctors is imperial as it is very important to provide the correct results of laboratory analyses to patients and also the laboratory staff must have the patient history for to correlate assessed tests [1].

Bilirubin is the by-product of heme catabolism. Normal metabolism of bilirubin involves its transport to the liver where it is conjugated to glucuronic acid, a process catalyzed by the enzyme bilirubin UDP-glucuronosyltransferase (bilirubin-UGT) [Figure 1]. The test for good liver "function" consists in assessing aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), conjugated bilirubin (Bc), and unconjugated bilirubin (Bu), lactate dehydrogenase (LDH), gama-glutamyltransferase (GGT), albumin, and prothrombin time. Of these tests, only the albumin, the albumin/total protein, the
bilirubin, and prothrombin time are actual indicators of regular hepatic function. For other functions of the liver, such as drug metabolism, nutrient storage, intermediary metabolism, glycosylphosphatidylinositol-anchored membrane proteins, such as ALP, GGT and 5'-nucleotidase, are the most useful markers in cholestatic liver injury.[2.3, 4]

There are many causes of hepatitis, including viruses (e.g., hepatitis A, B, and C), toxins (e.g., acetaminophen), alcohol, ischemia, Reye syndrome, and autoimmune diseases. Aminotransferase can often be increased by as much as 50 times the upper reference limit in acute viral, ischemic, and toxic hepatitis, whereas in alcoholic hepatitis the increases are generally <10-fold. The value for ALT is usually higher than for AST and this is most likely due to the exclusively cytoplasmic distribution of ALT and the longer half-life period in the blood – approx. 50 h, compared to approx. 16 h for AST. The exception is alcoholic liver disease, in which the AST/ALT ratio is often >2.

Regardless of the cause, chronic hepatitis is characterized by milder and fluctuating increases in the levels of aminotransferases (ALT and AST). Other hepatic causes for increased levels of aminotransferases include hemochromatosis, nonalcoholic fatty liver disease, and Wilson disease. [5, 6]

Genetic diseases which cause abnormalities in liver bilirubin levels were registered in 5% of the general population. Depending on the etiology, jaundices may include:

1. Jaundices by shunt (rare) – result of accelerated erythopoiesis due to bone marrow disorders which result in early destruction of immature red blood elements. (Reye Syndrome). [7]

2. Jaundices of production – appear as a result of massive hemolysis, due to corpuscular abnormalities (Hereditary Spherocytosis, Paroxysmal Hemoglobinuria Nocturne, Glucose 6 Phosphatase deficiency (G6PD)), or extra-corpuscular hemolysis of red blood cells (toxic syndromes, autoimmune anemia, hemoglobinopathies).

3. Congenital jaundice of transport – characterized by the decrease or absence of intracellular protein transport of bilirubin (lack of endothelial receptor for albumin in liver cells, lack of organic anion binding protein, lack of endoplasmic reticulum ligands for bilirubin molecules in liver cells).

4. Jaundice of conjugation – due to the reduced levels or absence of the uridil-glicuronil-transferase enzyme. The Gilbert Syndrome (GS) is caused by an approximately 70%-75% reduction in the glucuronidation activity of uridine-diphosphate-gluconosyltransferase isoform 1A1 (UGT1A1).

The gene that encodes UGT1A1 normally has a TATA promoter region which contains the allele A (TA6) TAA, while GS is most commonly associated with homozygous A (TA7) TAA alleles. [8] Lack of UGT1A1 in fetal hepatocytes causes Crigler Najar syndrome (CNS), a rare disorder affecting the level of conjugate bilirubin (Bc) CNS causes an inherited non-hemolytic jaundice, often leading to brain damage in infants. This syndrome is divided into two types: type I and type II. Unlike type I CNS, in type II, bilirubin levels are generally below 345 μmol/l (10 – 20.1 mg/dl); thus some type II cases are only detected later in life. Additionally, kernicterus is rare in type II, because unconjugated bilirubin constitutes the largest fraction of bile conjugates. [10].

5. Jaundices of secretion – the transport and proper conjugation of bilirubin in fetal hepatocytes does occur, but its delivery from the liver cells is blocked by the lipofuscin
pigment, through biliary obstruction, (Dubin
Johnson syndrome) or deposition of pigment
in the biliary intra-hepatic way (Rotor
Syndrome). Dubin-Johnson syndrome is a
very rare genetic disorder. In order to
transmit the inherited condition, one of the
parents must have a copy of the defective
gene. Mild jaundice, which may not appear
until puberty or adulthood is the only

Scope
The aim of this work was to identify and to
interpret the variability of Bilirubin values in
serum samples together with high values of
Triglycerides and variable values of
Cholesterol, to avoid the loss of congenital
liver syndromes in diagnosis of laboratory,
because of the interference of analytes in
samples of patients, which have presented
recently in a private laboratory for a routine
para-clinical control.

2. Material and Method

The study was conducted on 160 patients
with 10 major blood tests performed: (a) 5
Substrates/chemistries: cholesterol, total
bilirubin, conjugated bilirubin, un-conjugated
bilirubin as derivative test, triglycerides, and
(b) 5 enzymatic tests: aspartate
aminotransferase (AST), alanine
aminotransferase (ALT), alkaline
phosphatase (ALP), lactate dehydrogenase
(LDH), gamma-glutamyltransferase (GGT).
These tests were performed on a Roche
Hitachi 912 automatic analyzer.

Additionally, hepatic viral markers (Ag HBS,
Anti HCV) were analyzed on an Elisa
Analyzer, as well as hemograms with
differential count and reticulocytes, on a
Coulter Analyzer. Out of the 160 patients 70
were females (20-30 years, mean age= 26,
SD=2.6) and 90 males (25-36 years, mean
age 30, SD=2.8). All the equipment was
calibrated and validated prior to the testing.

The patients chosen for this study had not
had any food or drinks for at least 4 hours
prior to the blood sampling, had an estimated
alcohol intake of 50 mg or less, daily, and
had not taken any medications for at least
two months before the testing. Therefore,
none of these factors could have been the
cause of increased bilirubin levels. The
samples were kept at -20º C, in 100 µl
sample cups, in the dark – to avoid the light-
induced breakdown of bilirubin –, for no
longer than 5 days.

The interpretation of the liver tests was
correlated with the clinical conclusions of the
physicians who referred those patients to the
laboratory. In all cases, the physical
examination of the patients revealed no
physical abnormalities, a non-icteric sclera, a
flat, soft abdomen without organomegaly,
and no signs of swelling of the eyelids. The
patients’ history did not include any acute or
chronic liver diseases, or any cardiovascular,
chronic metabolic or endocrine diseases.

The controls and references interval
established on 120 known apparent health
patients, on each up parameters measured
with central IC, 95%. Quality Control
Samples was performed on control samples
for at least two levels, normal and high level.

The reference interval of laboratory presented
followed values: Total Bilirubin = 0.2-1
mg/dl, Un-conjugated Bilirubin =0.3-0.7
mg/dl, Conjugated Bilirubin = 0.0-0.3 mg/dl,
AST for females = 15-36 U/L, AST for
males 11-55 U/L, TGP for males=11-43
U/L and TGP for females =9-52 U/L, lactate
derhyrogenase (LDH=100–250 U/L),

360
Figure 1.

Enterohepatic circulation of bilirubin. The bilirubin conjugated to glucuronate is catalyzed by the enzyme Bilirubin UDP-glucuronyltransferase (bilirubin-UGT)

Gama-glutamyltransferase (GGT) = 11–50 U/L, Alkaline Phosphatase (ALP) = 50–170 U/L, Total Cholesterol = 109–220 mg/dl, [Table 1], HDL = 36–60 mg/dl, LDL = 48–130 mg/dl and Triglycerides = 53–145 mg/dl. [Table 2].

After a 1:5 dilution of the samples with high levels of triglycerides, only 2.8% of tests showed normal values of unconjugated bilirubin, (negative predictive value=66%) and 4% of tests had the same high results of unconjugated bilirubin, pointing toward a congenital liver syndrome, (positive predictive value=72%).

In a parallel study on a sample of 120 apparently healthy – no evidence of any pediatric disease – adolescent patients (ages 12-18), all results were normal and only 6 (5%) of the cases showed a high unconjugated bilirubin, suggesting Gilbert's syndrome, which must be confirmed by cytogenetic studies.

3. Results:
Out of the 160 patient sample, 90% (n=144) were healthy patients with normal blood-work. In 6.8% cases, there was an increase in the levels of total bilirubin (average = 1.4-7.7 mg/dl, mean value = 1.99 mg/dl, interval of reference = 0.2-1.0; CV = 0.13; p = 0.02 ) and an increase of unconjugated bilirubin (average = 1.0-4.9 mg/dl, mean value = 1.45 mg/dl, interval of reference = 0.3-0.7 mg/dl; CV=0.18; p=0.01); these results were accompanied by normal levels of liver enzymes, but high levels of triglycerides, with values of over 243 mg/dl in samples without macroscopic aspect of turbidity.
Table 1.
Results of investigated cases for Cholesterol in serum samples

<table>
<thead>
<tr>
<th>CHOLESTEROL</th>
<th>Lipid metabolism disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>mg/dL</td>
</tr>
<tr>
<td>&lt;200</td>
<td>44 case = 26.4%</td>
</tr>
<tr>
<td>≥200 – 300</td>
<td>11 cases = 6.6%</td>
</tr>
<tr>
<td>&gt;300</td>
<td>5 cases = 3%</td>
</tr>
</tbody>
</table>

Desirable cholesterol level: < 5.2 mmol/L (<200 mg/dL),
Borderline high cholesterol: < 5.2–6.2 mmol/L (200 – 239 mg/dL),
High cholesterol: ≥6.2 mmol/L (≥240 mg/dL),
Cholesterol reference range reagent = 60–240 mg/dL
Cholesterol proper reference range = 109–220 mg/dL

Externally Quality Control

VALUE = 128 mg/dL
Z-Score = 0.80
BIAS = 4.54

Precision Cholesterol assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>MEAN</th>
<th>cv %</th>
<th>Within-run</th>
<th>MEAN</th>
<th>cv %</th>
<th>Between-run</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum</td>
<td>220</td>
<td>0.8</td>
<td>204</td>
<td>210.1</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Precinorm U</td>
<td>167</td>
<td>1.0</td>
<td>182</td>
<td>114.7</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Precipath U</td>
<td>129.4</td>
<td>0.7</td>
<td>126.7</td>
<td>126.7</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.
Results of investigated cases for Triglycerides in serum samples

<table>
<thead>
<tr>
<th>TRIGLYCERIDES</th>
<th>Lipid metabolism disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>mg/dL</td>
</tr>
<tr>
<td>&lt;200</td>
<td>No</td>
</tr>
<tr>
<td>≥200 – 300</td>
<td>Yes if HDL – cholesterol &lt; 0.9 mmol/L (&lt;35 mg/dL)</td>
</tr>
<tr>
<td>&gt;300</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Triglycerides: reference range reagent = 50–120 mg/dL
Expected range: < 2.26 mmol/L (<200 mg/dL)
Triglycerides: proper reference range = 53–145 mg/dL

Quality External Control

Value = 130 mg/dL
Z-Score = 0.14
BIAS% = 1.43

Precision Triglycerides assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>MEAN</th>
<th>cv %</th>
<th>Within-run</th>
<th>MEAN</th>
<th>cv %</th>
<th>Between-run</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum</td>
<td>142</td>
<td>1.5</td>
<td>224.1</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precinorm U</td>
<td>105</td>
<td>0.9</td>
<td>108.8</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipath U</td>
<td>137.2</td>
<td>0.9</td>
<td>130.5</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.

DISEASES AND CONDITIONS WITH PRE-HEPATIC, HEPATIC AND POST-HEPATIC JAUNDICE

<table>
<thead>
<tr>
<th>Pre-Hepatic Jaundice</th>
<th>Hepatic Jaundice</th>
<th>Post-Hepatic Jaundice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolytic anemia due to G6PD deficiency, Hereditary spherocytosis, Sickle cell anemia, Erythroblastosis fetal Hemoglobinopathies, Paroxistic-Hemoglobinuria Nocturne, Thalassemia, Immune hemolytic anemia, Transfusion reaction Idiopathic aplastic anemia Secondary aplastic anemia Non-immune hemolytic anemia, drug-induced jaundice</td>
<td>Chronic liver diseases Chronic Hepatitis</td>
<td>Biliary stricture with duct obstructions</td>
</tr>
<tr>
<td></td>
<td>Chronic Hepatitis</td>
<td>Cholecystitis</td>
</tr>
<tr>
<td></td>
<td>Wilson's disease</td>
<td>Cholelithiasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cholangiocarcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bile duct obstruction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wilson's disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gilbert's disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dubin-Johnson syndrome</td>
</tr>
</tbody>
</table>

1*: Drug-induced jaundice
allopurinol, anabolic steroids, some antibiotics, antimalaria medications, azathioprine, chlorpropamide, cholinergics, codeine, diuretics, epinephrine, meperidine, mehtotrexate, methylidopa, MAO inhibitors, morphine, nicotinic acid, birth control pills, phenothiazines, quinidine, rifampin, steroids, sulfonamides, and theophylline or decrease bilirubin measurements as barbiturates, caffeine, penicillin, and high-dose salicylates such as aspirin.[2]

Table 4

Variability of Bilirubin Tests in Hepatic Jaundice*, Acute Hepatitis, Active Chronic Hepatitis, Chronic Persistent Hepatitis, Cirrosis, Colestatic Syndromes, Liver Malignant Diseases or Metastasis.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>TOTAL BIL</th>
<th>DIRECT BIL</th>
<th>INDIRECT BIL</th>
<th>AST; ALT; LDH; GGT; ALP</th>
<th>RETIC. COUNT</th>
<th>VIRAL MARKERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Status</td>
<td>0.5-1.3</td>
<td>0.0-2</td>
<td>0.3-1.1</td>
<td>N</td>
<td>0.5-1.2%</td>
<td>Negative</td>
</tr>
<tr>
<td>Pre-hepatic Jaundice</td>
<td>↑</td>
<td>N</td>
<td>↑</td>
<td>N or ↑</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Hepatic Jaundice</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑↑</td>
<td>N</td>
<td>±</td>
</tr>
<tr>
<td>Gilbert Syndrome</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Cligler-Najjar Syndrome</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Dubin Jonson Syndrome</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Pos-hepatic Jaundice</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>AST ↑; ALT↑; LDH ↑; GGT↑; ALP↑;</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>Hemolitic Jaundice</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↑ AST, ALT, LDH;</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Interference of Tryglicerides</td>
<td>↑</td>
<td>N</td>
<td>↑</td>
<td>N</td>
<td>N</td>
<td>-</td>
</tr>
</tbody>
</table>
4. Discussions

Spectral interference and laboratory results show that the choice of the methods is very important, but equally important is the way these methods are adapted for clear and accurate testing. By looking into these conditions carefully, it is sometimes necessary to find a method for correcting any interference problems.

If the different factors that are analyzed cause erroneous results, then the user can choose a sample blank or a secondary wavelength, and so, the effect of the interference can be better kept under control. The most notable issue observed during the study was the poorly effective bichromatic procedure: the choice of the secondary wavelength did not always prove useful, and thus, the “corrections” of samples often had to be done by an effective dilution.

Depending on the environmental factors, high values of bilirubin with mild jaundice can affect the liver, can cause pregnancy symptoms, can worsen by ingestion of alcohol and pills (especially contraceptives), and by complications of infections and strenuous physical activity. [12]. Table 3

Elevated values of triglycerides, correlated with increased levels of total bilirubin (in the presented cases), can allow for a differential diagnosis with liver congenital syndromes, by measurement of isolated high indirect bilirubin.

Gilbert’s syndrome produces an elevated level of unconjugated bilirubin in the bloodstream but normally has no serious consequences and is quite frequent in the population (5-6% of the population). Mild jaundice may appear under conditions of exertion, stress, fasting, and infections but is usually asymptomatic. It has been reported that GS may contribute to an accelerated onset of neonatal jaundice, especially in the presence of increased hemolysis due in diseases like G6PD deficiency. The enzyme which is defective in GS (UGT1A1) is also responsible for the liver's ability to detoxify certain drugs. [9].

High values of total bilirubin may be a sign of Crigler-Najjar syndrome, Gilbert's disease, hemolytic anemia, hepatitis, or physiological jaundice (normal in newborns), and thus, additional tests may be performed for a better differential diagnosis. Hemolysis can be excluded by a full blood count, lactate dehydrogenase levels, and the absence of reticulocytosis; elevated reticulocytes in the blood would usually be observed in hemolytic anemia. Increased values of conjugated bilirubin would indicate: cirrhosis, Dubin-Johnson syndrome (very rare), hepatitis, intra-hepatic cholestasis (buildup of bile in the liver). [11] Table 4

High values of triglycerides can increase the level of total bilirubin, un-conjugated bilirubin, and conjugated bilirubin; in this case a differential diagnosis must be performed, considering the various congenital syndromes that can cause elevated bilirubin levels.

The Hitachi analyzer – which uses a photocolorimetric system – measures bilirubin using a wavelength of 546 nm (540-560 nm) and by PAP enzymatic method; the cholesterol and triglycerides levels are measured using a 505 nm wavelengths, (480-520) nm (same spectral region), exhibiting the most interference. In order to avoid misleading results, three approaches can be used: (1) the selection of analysis methods that give minimal interference. (2) The use of computerized techniques: common tools include using mathematical index delta checks, which can detect unlikely changes from previous results, and (3) the use of self-verification rules to detect medically unlikely results. If the approaches are selected
correctly, they should draw attention upon the samples with results that are likely misleading. [13].

Furthermore, the common cases of interference must be recorded and their potential clinical impact must be studied, in order to understand the various pre-analytical sources of error which can lead to discrepant results. It is also very important that the laboratorian can identify the cases of interference caused by exogenous factors, such as drugs and herbal medicine, and implement strategies that can help solve these testing issues (such as the ones mentioned above).

Many of today's highly automated laboratory instruments have built-in mechanisms that help laboratories identify sample integrity, identify the test results that are outside specified reference range, and to identify outliers which require further investigation. These tools help us ensure accurate and precise testing, but cannot always pick up the testing errors caused by interferences. The cause of interference and the solution are both hard to identify, and so, a very good technique is required of the laboratory personnel.

5. Conclusions:

High values of triglycerides can increase level of total bilirubin, Bu, Bc, and in laboratory it is needed to make a differential diagnosis with a form of hepatic congenital syndromes with isolated high bilirubin values.

The pre-analytical sources of errors can lead to discrepant results and for this we must implement strategies that can help to manage the test interferences. The laboratory physician must detect the common interferences and he must make the review of potential clinical impact.

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Author Contributions:
All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest:
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7. REFERENCE