Acyl carnitine and Amino Acids Profile in Biliary Atresia versus Cholestasis in Paediatric Patients; Comparative Study.

Ahmed A Raouf¹, Mohamed A El Gendy³, Hatem M El Sebaay², Ashraf Y El-Fert¹ and Israa M Ismail¹

¹Clinical Biochemistry Department, National Liver Institute, Minoufia University, Egypt.
²Medical Biochemistry Department, Faculty of Medicine, Minoufia University, Egypt
³Pediatric Department, National Liver Institute, Minoufia University, Egypt

Israataher@ymail.com

Abstract: Objective: compare different levels of amino acids and acyl carnitines profile to give an insight about metabolic pathways in cholestasis and biliary atresia (BA) in pediatric patients compared to controls. Background: Early detection is the most effective way to improve the clinical outcome of (BA). Emerging metabolomics as amino acid and acyl carnitine provide a powerful platform for discovering new biomarkers and biochemical pathways to improve early diagnosis. Methods: This study includes 35 BA patients, 35 Neonatal Cholestasis (NC) rather than BA patients and 35 healthy controls. Liver function tests, abdominal ultra-sonography, liver biopsy were done to all subjects. Amino acid and acyl carnitine assay using high performance liquid chromatography tandem mass spectrometry (LC MS/MS). Results: The data revealed a statistical significance increase in methionine, glutamate, citrulline and AAA and decrease in BCAA and fisher ratio in both studied groups compared to control, also there was increase in ornithine and decrease in glycine amino acid in BA group compared to control and increase in Arginine in NC rather than BA group. Our results, also indicated a statistical significant increase in both patients groups compared to control regarding to C0, C2, C3, C4, C5, C5DC, C6, C12, C14, C16 and C18; while fisher ratio, simplified fisher ratio, C4 and C18 showed statistical difference between the two studied groups. Conclusion: The findings of the present study indicate that there is a metabolic profile shift of amino acid and acyl carnitine in BA from NC detected by LC/MSMS. They can be potentially developed into a useful diagnostic tool and contributing towards understanding of the disease mechanism.


Keywords: Biliary Atresia, Neonatal Cholestasis, Amino acids, Acylcarnitin.

1. Introduction

Cholestasis is the failure of bile to reach the duodenum, which may be caused by pathology anywhere between the hepatocyte and the ampulla of Vater. Neonatal cholestasis (NC) is defined as persistence of direct bilirubin more than 20% of total serum bilirubin for more than 14 days. Cholestasis in infancy is caused by a wide range of conditions including obstructive, infectious, metabolic and genetic causes with variable incidence (1).

Biliary atresia (BA) is a progressive destruction of the extra hepatic and intrahepatic bile ducts, with scarring and obliteration with mostly unknown etiology and pathogenesis (2). BA constitutes 25–34% of all neonatal cholestasis and more than 90% of obstructive cholestasis cases. It is important to be differentiated from other causes of neonatal cholestasis as early as possible for the success of Kasai portoenterostomy. If portoenterostomy is not successful or not performed, liver transplantation is the only life-saving alternative (3). BA is the indication for 75% of all liver transplants performed in children below 2 years (4).

If left untreated, these disorders produce biliary cirrhosis and eventual hepatic failure; thus it is anticipated that several biochemical metabolic pathways would be affected through the disease progression (5). As it is still difficult to differentiate BA from other causes of neonatal cholestasis due to limitations in conventional approaches, noninvasive tests have been developed to facilitate such diagnosis (6).

As the liver plays a central role in amino acid metabolism, it is of great importance to investigate the metabolic profiles in BA and other causes of NC (7). Carnitine plays an important role in fatty acid oxidation and energy production. It helps in β- oxidation of fatty acids by entering into the mitochondria. The carnitine bound with acyl-CoA to form acyl carnitine. Experiments indicate that biliary export was a major route for acyl carnitine clearance (8, 9).

In the previous studies, there were different amino acid and acyl carnitine metabolite profiles corresponding to the different forms of liver disease. However there were only a few studies on BA using liquid Chromatography-Mass Spectrometry based methods to investigate the acyl carnitine profiles (7).

Indeed amino acid profile in cholestatic patients showed abnormal amino acid pattern (5). Also in biliary
atresia, Byrd and associates1993 showed altered amino acid profile, especially if complicated with liver cirrhosis (10). Nevertheless, such changes in serum amino acid could not be detected by (Matthews and coworkers, 2002)(11).

Therefore, we set out to evaluate these alterations sensitivity and specificity in differentiate BA from other causes of NC

2. Subjects and methods
2.1. Study population
This study was conducted on 105 subjects including 35 diagnosed as BA patients and 35 NC rather than BA. They were presented to pediatric Department, National Liver Institute, Minoufia University in the period from July 2012 to July 2013. Diagnosis was based on clinical examination, laboratory tests, Ultrasound and liver biopsy in addition to a control group comprised of 35 age and gender matched infants.

All patients and control groups were subjected to the following:
1- Full history taking.
2- Complete physical examination.
3- Abdominal ultrasonography.
4- Liver biopsy.
5- Laboratory investigation including:
   • Liver function tests: Total and direct serum bilirubin (13), the cholestatic enzymes alkaline phosphatase (ALP) and gamma glutamyle trans-peptidase (GGT), aspartate transaminase (AST), alanine transaminase (ALT)(14), total protein (TP) and serum albumin were measured using the Beckman Coulter (Synchron CX 9 ALX) Clinical Auto analyzer (Beckman Instruments, Fullerton, CA, USA).
   • Amino acid and acyl carnitine assay using high performance liquid chromatography tandem mass spectrometry (LC MS/MS).

A written consent was obtained from the guardian of each individual and the protocol was approved by the ethical committee of Minoufia University Faculty of Medicine and National Liver Institute

2.2. Sample collection:
a. Blood sample collection :
A volume of 4ml of venous blood were collected from all subjects included in this study the sera were separated used for measurement of liver function tests.
b. Sample collection for amino acid and acyl carnitine assay:
The blood sample was taken from the heel of the infant using Guthrie card made of Whatman 903 filter paper purchased from (GE Healthcare, NJ, USA ).

The blood spots were dried for 4 hours on a dry, horizontal and non-absorbent surface at ambient temperature.

2.3. Amino acid and acyl carnitine assay using HPLC
Tandem mass spectrometry (LC/MS/MS):
The blood spots were analyzed for acyl carnitines and amino acids by triple-quadruple tandem mass spectrometer (ACQUITY UPLC H-Class. Waters corporation, MA, USA) with a positive electrospray ionization probe, using MassChrom® Amino acids and Acylcarnitines from Dried Blood / Non derivatised kit (Chromsystems Instruments & Chemicals GmbH, München, Germany).

Assay procedure:-
A 3 mm dried blood spot disk was punched out of the filter card into a well of v-bottomed microtiter plate 100 µl of the reconstituted internal standard(with 25 ml of Extraction Buffer provided by the kit) was added to each dried blood spot disk. Afterward, the plate was agitated and supernatant was transferred into a new v-bottomed well plate that would be sealed with protective sheet.

Now the samples were ready for Injection whereas 10 µl of the elute was injected into the LC-MS/MS system.

Equipment and instrument parameters:
The flow of mobile phase was adjusted to 200(µl/min) then at 0.25 min it reduced to 20(µl/min) and then up to 600 (µl/min) at 1.25 min to be reduced again to 200 (µl/min) whereas the scan time window of the tandem MS system has to be set in the period of time approximately 1.25 min.

2.4. Statistical methods
Data were statistically analyzed using SPSS (statistical package for social science) program version 13 for windows for all the analysis. P value < 0.05 was considered statistically significant.

3. Results
Table (1) shows that all the studied groups are homogenous regarding age and gender, as there is no statistically significant difference between BA and NC rather than BA groups and control groups.

Table (2) shows a significant increase in ALT,AS, ALP, TB, DB and GGT in studied patients groups compared with control group on the other hand there is a significant decrease in TP in studied patients group and GGT shows significant increase in BA group compared to NC rather than BA group.

Table (3) shows significant increase levels of methionine, citrulline and glutamate in BA and NC rather than BA groups compared to control group on the other hand there is a significant decrease in TP in studied patients group and GGT shows significant increase in BA group compared to NC rather than BA group.

Table (3) shows significant increase levels of methionine, citrulline and glutamate in BA and NC rather than BA groups compared to control; and valine showed significant decrease in both studied patients groups compared to control group; however leucine/Isoleucine showed significant decrease only in NC rather than BA group compared to control; Phenylalanine shows significant increase in both studied patients groups compared to control but
tyrosine showed significant increase in NC rather than BA group compared to control; glycine shows significant decrease but ornithine shows significant increase in BA group compared to control group; while arginine has significant increase in NC rather than BA group compared to control group. No significant alteration in level of aspartate, alanine and proline in the two studied groups compared to control.

Table (1): Age and gender differences between studied groups

<table>
<thead>
<tr>
<th>The studied groups</th>
<th>Test of significance</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biliary atresia N = 35</td>
<td>Control N = 35</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age/days</td>
<td>74.94±61.44</td>
<td>72.26±69.65</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td>Male</td>
<td>20 57.1</td>
<td>24 68.6</td>
</tr>
<tr>
<td>Female</td>
<td>15 42.9</td>
<td>11 31.4</td>
</tr>
</tbody>
</table>

*p-value >0.05 = non-significant.

In table (4) there is statistical significant decrease in branched chain amino acid and Fisher ratio in both studied patients groups compared to control group. While simplified fisher ratio decreases and aromatic amino acid increases in NC rather than BA compared to control. There is statistical significant between BA and NC rather than BA groups regarding fisher ratio, simplified fisher ratio and phenylalanine/tyrosine ratio.

Table (2): Differences between BA& NC rather than BA and Control regarding liver function tests.

<table>
<thead>
<tr>
<th>The studied groups</th>
<th>Mann Whitney U</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biliary atresia N = 35</td>
<td>Control N = 35</td>
<td></td>
</tr>
<tr>
<td>AST (IU/L) N = 35 (Mean ± SD)</td>
<td>221.26±95.72</td>
<td>0.06</td>
</tr>
<tr>
<td>ALT (IU/L) N = 35 (Mean ± SD)</td>
<td>150.65±90.47</td>
<td>7.20</td>
</tr>
<tr>
<td>ALP (IU/L) N = 35 (Mean ± SD)</td>
<td>440.91±182.38</td>
<td>15.83±7.84</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl) N = 35 (Mean ± SD)</td>
<td>10.86±4.51</td>
<td>1.75</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dl) N = 35 (Mean ± SD)</td>
<td>8.28±3.06</td>
<td>1.59</td>
</tr>
<tr>
<td>Albumin (g/dl) N = 35 (Mean ± SD)</td>
<td>3.33±0.48</td>
<td>1.46</td>
</tr>
<tr>
<td>Total protein (g/dl) N = 35 (Mean ± SD)</td>
<td>5.17±0.63</td>
<td>1.45</td>
</tr>
<tr>
<td>GGTT (IU/L) N = 35 (Mean ± SD)</td>
<td>980.17±700.23</td>
<td>4.66</td>
</tr>
</tbody>
</table>

* = t-test; 1 = comparison between group of biliary atresia and group without biliary atresia
2 = comparison between of biliary atresia and control
3 = comparison between group without biliary atresia and control
Table (3): Statistical comparison between the three studied groups as regards amino acids (µmol/l)

<table>
<thead>
<tr>
<th>The studied groups</th>
<th>Neontal Cholestasis</th>
<th>Control</th>
<th>Mann Whitney U</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biliary atresia N = 35</td>
<td>Non biliary atresia N = 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>22.02±5.66</td>
<td>29.69±18.52</td>
<td>15.20±4.65</td>
<td>1.13</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>126.28±75.47</td>
<td>154.19±68.67</td>
<td>104.36±43.99</td>
<td>1.97</td>
</tr>
<tr>
<td>Ornithine</td>
<td>147.56±52.77</td>
<td>143.48±48.98</td>
<td>133.91±74.05</td>
<td>0.32</td>
</tr>
<tr>
<td>Citruline</td>
<td>18.44±8.96</td>
<td>18.97±7.69</td>
<td>12.07±4.02</td>
<td>0.63</td>
</tr>
<tr>
<td>Valine</td>
<td>75.06±23.58</td>
<td>74.42±14.16</td>
<td>91.89±35.37</td>
<td>2.07</td>
</tr>
<tr>
<td>Glycine</td>
<td>263.94±103.79</td>
<td>282.74±137.52</td>
<td>338.80±158.66</td>
<td>0.13</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>52.34±33.41</td>
<td>44.18±14.02</td>
<td>35.91±9.03</td>
<td>0.80</td>
</tr>
<tr>
<td>Aspartate</td>
<td>45.11±35.26</td>
<td>48.71±23.20</td>
<td>51.48±26.67</td>
<td>1.38</td>
</tr>
<tr>
<td>Alanine</td>
<td>223.24±71.13</td>
<td>238.76±107.93</td>
<td>264.63±118.93</td>
<td>0.24</td>
</tr>
<tr>
<td>Arginine</td>
<td>15.82±12.03</td>
<td>21.17±12.21</td>
<td>12.50±10.60</td>
<td>1.98</td>
</tr>
<tr>
<td>Proline</td>
<td>148.69±51.94</td>
<td>147.47±57.75</td>
<td>155.92±64.37</td>
<td>0.37</td>
</tr>
<tr>
<td>Leucine/Isoleucine</td>
<td>116.19±34.78</td>
<td>102.77±31.57</td>
<td>133.84±46.90</td>
<td>1.63</td>
</tr>
<tr>
<td>Glutamate</td>
<td>351.77±110.11</td>
<td>319.06±147.82</td>
<td>126.38±33.52</td>
<td>1.68</td>
</tr>
</tbody>
</table>

1 = comparison between group of biliary atresia and group without biliary atresia  2 = comparison between of biliary atresia and control  3 = comparison between group without biliary atresia and control

Table (4): Statistical comparison between the three studied groups as regards amino acids ratio

<table>
<thead>
<tr>
<th>The studied groups</th>
<th>Neontal cholestasis</th>
<th>Control</th>
<th>Mann Whitney U</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biliary atresia N = 35</td>
<td>Non biliary atresia N = 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branched Chain</td>
<td>191.26±55.37</td>
<td>177.19±51.47</td>
<td>225.73±65.77</td>
<td>1.10</td>
</tr>
<tr>
<td>Amino Acids (BCAA)</td>
<td></td>
<td>2.37</td>
<td>3.44</td>
<td></td>
</tr>
<tr>
<td>Non -Branched Chain Amino Acid (NBCAA)</td>
<td>1214.58±315.73</td>
<td>1220.36±342.75</td>
<td>1095.70±380.44</td>
<td>0.07</td>
</tr>
<tr>
<td>Essential amino acids</td>
<td>265.62±76.78</td>
<td>251.06±63.11</td>
<td>276.84±69.40</td>
<td>0.87</td>
</tr>
<tr>
<td>Non-essential amino acids</td>
<td>1174.86±301.02</td>
<td>1212.11±364.84</td>
<td>1054.08±342.29</td>
<td>0.47</td>
</tr>
<tr>
<td>Aromatic amino acids (AAA)</td>
<td>178.62±92.31</td>
<td>198.37±73.10</td>
<td>140.26±47.57</td>
<td>1.33</td>
</tr>
<tr>
<td>Fisher ratio</td>
<td>1.22±0.41</td>
<td>0.97±0.35</td>
<td>1.76±0.74</td>
<td>2.81</td>
</tr>
<tr>
<td>Simplified Fisher ratio</td>
<td>2.04±1.31</td>
<td>1.31±0.56</td>
<td>2.65±1.90</td>
<td>2.92</td>
</tr>
<tr>
<td>Phynyl alanine/ tyrosine ratio</td>
<td>0.57±0.46</td>
<td>0.33±0.14</td>
<td>0.43±0.29</td>
<td>2.40</td>
</tr>
</tbody>
</table>

1 = comparison between group of biliary atresia and group without biliary atresia  2 = comparison between of biliary atresia and control  3 = comparison between group without biliary atresia and control

* = t-test  ** = Mann Whitney U test

**Degraded text and symbols due to formatting issues.**
Table (5): Statistical comparison between the three studied groups as regards acyl carnitines(µmol/l).

<table>
<thead>
<tr>
<th>The studied groups</th>
<th>Mann Whitney U</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutonal cholestasis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biliary atresia N = 35</td>
<td>Non-biliary atresia N = 35</td>
</tr>
<tr>
<td>free carnitine (C0)</td>
<td>39.23±23.31</td>
<td>33.72±19.99</td>
</tr>
<tr>
<td>Acetyl carnitine (C2)</td>
<td>29.89±19.31</td>
<td>33.54±20.60</td>
</tr>
<tr>
<td>propionylcarnitine (C3)</td>
<td>1.62±1.28</td>
<td>1.50±0.88</td>
</tr>
<tr>
<td>Butyrylcarnitine(C4)</td>
<td>0.24±0.26</td>
<td>0.30±0.16</td>
</tr>
<tr>
<td>Isovalerylcarnitine(C5)</td>
<td>0.17±0.08</td>
<td>0.20±0.07</td>
</tr>
<tr>
<td>Glutarylcarnitine(C5DC)</td>
<td>0.22±0.09</td>
<td>0.25±0.09</td>
</tr>
<tr>
<td>Hexanoylcarnitine(C6)</td>
<td>0.09±0.10</td>
<td>0.17±0.48</td>
</tr>
<tr>
<td>Octanoylcarnitine(C8)</td>
<td>0.08±0.10</td>
<td>0.06±0.03</td>
</tr>
<tr>
<td>Decanoylcarnitine (C10)</td>
<td>0.12±0.08</td>
<td>0.10±0.05</td>
</tr>
<tr>
<td>Dodecanoylcarnitine (C12)</td>
<td>0.08±0.05</td>
<td>0.08±0.04</td>
</tr>
<tr>
<td>Tetradecanoylcarnitine(C14)</td>
<td>0.39±0.26</td>
<td>0.40±0.32</td>
</tr>
<tr>
<td>Hexadecanoylcarnitine(C16)</td>
<td>1.97±0.93</td>
<td>2.39±1.39</td>
</tr>
<tr>
<td>Octadecanoylcarnitine (C18)</td>
<td>0.92±0.56</td>
<td>1.60±1.30</td>
</tr>
</tbody>
</table>

1 = comparison between group of biliary atresia and group without biliary atresia; 2 = comparison between of biliary atresia and control; 3 = comparison between group without biliary atresia and control.

4. Discussion
The present study was conducted on 35 BA and 35 NC rather than BA patients recruited from the pediatric inward department from National Liver Institute in Minoufia University, during the period from July 2013 to July 2013. The mean age of presentation BA patients in this study was nearly 75 days; while those of NC rather than BA their mean age was nearly 78 days.

Bazlul Karim and Kamal (2005) (15) showed that age of presentation of NC was 75 day, while Bellomo-Brandao et al., (2010) (16) reported that the median age of infants in the BA group was over 60 days (77 days) of life and higher than that in the NC group. Data published in Brazil show that in the majority of cases, the diagnosis of BA is made after 8 weeks of life (17).

Also Rashed et al., (2013) (18) reported that the mean age of presentation in his study on BA was 68 day similar to that reported by the Biliary Atresia Research Consortium (BARC) of 9 referral centers in the United States where, between 1999 and 2003, the mean age at diagnosis was 61 days (19).
Even though most of the children were symptomatic since birth, so it seems that there has been no improvement in the age at diagnosis of BA over the past 15 years. Indeed, there is a concerning trend toward an increase, with many patients still being diagnosed far too late (Wadhwani et al., 2008) (20).

Although it is reported that females are slightly more affected with BA than males (21); in this study males represent nearly 57% while females represented about 43% in both BA and non BA group and that in parallel with Shah et al., (2012) (19) who showed in his study on BA patients that males represented 75% compared to 25% are females.

However, Lee and Chai, (2010) (21) and Osuoji et al., (2013)(22) demonstrated BA was more common among females when compared to other etiologies of NC as 57% in BA patients were females compared to 38% in other causes of NC. The discrepancy in the previous results compared to the current study could be attributed to the relatively small number of patients included in this study.

The liver biochemical profile: ALT, AST, γ-GT, ALP, TB and DB were significantly higher while serum level of TP is significantly lower in patients groups (BA and NC rather than BA) compared to control group. In addition, there was no statistically significant difference between patients groups regarding all these studied parameters but γ-GT being with higher values in BA group than NC group.

Our results agreed with Abdalla et al., (2013) (23) and Zhao et al., (2014)(7) who reported on his study that no statistical significant difference between BA patients and non BA patients regarding AST, ALT, ALP and TB except for GGT showed that significant increase in BA patients.

Regarding amino acids, the present study also revealed that there are significant increase levels of methionine, citrulline and glutamate in BA and NC rather than BA groups compared to control with no statistical difference between patients groups.

These results match these observed in earlier studies by Abukawa et al.,24 (2001), Zhang et al.,25 (2008) and Steinbach et al.,26 (2008)(24-26), who reported that plasma levels of amino acids citrulline and methionine were significantly greater, in patients of cholestatic jaundice during neonatal mass screening.

Methionine is the single essential sulfur containing amino acid; its metabolism is mainly in the liver playing important role in methyl group metabolism (27,28). In liver disease there is reduced metabolism of methionine and hyper- methioninemia and as vicious circle disrupted methionine metabolism lead to hepatic dysfunction which caused by aberrant methyl group flux (29-31).

While Citrulline is an intermediate in urea cycle (32) and liver takes up gut-derived citrulline, which limits the release of citrulline to the systemic circulation (33). Elevated plasma citrulline was an important biochemical marker of neonatal cholestasis caused by citrin deficiency (NICCD) which one of the causes of neonatal cholestasis (34).

On the other hand Glutamate is one of the most abundant amino acids in liver, kidney, skeletal muscle and brain (35). It has important role in case of liver dysfunction as liver cells fail to get rid of toxic ammonia, glutamate detoxifies it by an alternative pathways through formation of nontoxic glutamine from ammonia and glutamate which takes place mainly in brain and muscle (36).

In the present study there is significant decrease of overall branched chain amino acid (BCAA) in both groups (BA and NC rather than BA) compared to control group. The BCAA valine showed significant decrease in BA and NC rather than BA groups compared to control group; however the other BCAA leucine/ Isoleucine showed significant decrease only in NC rather than BA group compared to control but none such significance decrease was detected in BA group compared to control.

The diversity of serum level of BCAA could explained by the earlier studies on leucine and valine amino acids that reported that although both have similar patterns of metabolism, the body maintains valine at a higher free blood concentration than leucine for similar overall rates of oxidation (37). However knowledge about isoleucine is scarce (38).

Although advances in the management of children with neonatal cholestasis have enabled part of them to survive with their native livers, nearly all patients become cirrhotic (39). So this could be explanation of most of manifestations of chronic liver disease and its metabolic impacts o have appeared in cases of the current study.

These study results support findings in previous literature by (39) who reported significance decrease in serum level of valine, isoleucine and leucine in primary biliary cirrhosis. Also, earlier studies by Morgan et al., (1982), Delgado Dominguez et al., (1998) and Tajiri and Shimizu, (2013) (40-42) revealed significant decrease in serum concentration of BCAAs in patients with chronic liver diseases.

Multiple lines of evidence have shown that the main cause of the BCAA deficiency in liver cirrhosis is their consumption in skeletal muscle for synthesis of glutamate, which acts as a substrate for ammonia detoxification to glutamine (43).

In the present study there is significant increase in phenylalanine in both patients groups compared to control group but tyrosine showed significant increase in NC rather than BA group compared to control but
non such significance increase in BA group compared to control group, this study also reveals a trend in of rising serum levels of overall aromatic amino acids (AAA) which was with statistically significance detected only between NC rather than BA group and control group.

These findings matched with Shigematsu et al., (2002) (43) study which showed that tyrosine was elevated in neonatal cholestasis patients and Ohura et al., (2003) (44) who reported significant hyper phenylalaninaemia in neonatal cholestatic patients. Also Tajiri and Shimizu, (2013) (42) revealed that concentrations of the aromatic amino acids (AAAs) phenylalanine and tyrosine are increased significantly in patients with liver diseases which indicates predominantly cellular damage (42).

Phenylalanine is hydroxylated to tyrosine by the enzyme phenylalanine hydroxylase which is present in liver and kidney (31). In neonates, it has been suggested that phenylalanine hydroxylation is limited and can result in elevated phenylalanine and suboptimal tyrosine concentrations (29). However, more significant elevation of tyrosine in NC rather than BA group compared to BA group could be explained by tyrosenemia by itself is a cause of neonatal cholestasis (45).

In present study there is significant decrease in glycine in BA group compared to control.

While very little was found in literature about glycine in cholestasis, study by Morgan et al., (1982) (40) carried on patient with chronic liver disease showed reduced concentrations of plasma glycine level.

Glycine accomplishes important function for regulate the synthesis of the bile acid (46). In humans, greater than 95% of the biliary bile acids are conjugated with glycine or taurine amino acids, this occurs in liver through enzymatic reactions (47).

In present study ornithine showed significant increase in BA group compared to control group; while arginine had significant increase in NC rather than BA group compared to control group.

Prior little reviews had studied these amino acids, in Delgado Dominguez et al., (1998) and Ko et al., (2007) (41, 48) amino acid analyses showed that arginine were significantly elevated in all NC patient enrolled in his study. On the other hand Byrd et al., (1993) (10) revealed in his study that there was significant increase in ornithine level in BA comparing to control. Also, Nagasaka et al., (2009) (49) showed high levels of ornithine in liver disease patients.

The observed result of increase arginine in NC rather than BA group could attributed to hyperarginemia disease which not very uncommon to be presented in neonatal period as NC, however its typical presentation in early childhood. It is inborn error of metabolism due to arginase deficiency, which is the final enzyme of the urea cycle catalyzes the conversion of arginine to urea and ornithine (50).

Ornithine is a non-essential amino acid derived from the breakdown of arginine during urea cycle (51). As this amino acid participates in the urea cycle and these changes suggest impairment in the urea cycle (7).

The present study revealed no significant alteration in level of aspartate, alanine and proline in the two studied groups compared to control.

Several studies had reported varying observation concerning these three amino acids mainly showed no difference between patient and control group as those had done by Mata et al., (1975) and Shaw et al., (1984) (52, 53) who revealed no change in proline level between studied groups and Mukherjee et al., (2010) (51) study that showed no change in alanine level in the patients group compared to control group.

In contrary, Morgan et al., (1982) (40) showed that patients with primary biliary cirrhosis had increased concentrations of aspartate. On the other hand, other studies reported increased level of plasma proline in liver disease patients (54, 55).

The current study showed significant decrease in phenylalanine / tyrosine ratio in NC rather than BA group compared to BA group. This could be explained by increased tyrosine level in non BA group in the present study and need to be confirmed by larger sample size.

Phenylalanine/tyrosine ratio expressed the conversion of phenylalanine to tyrosine as it is thought to be exclusively located in liver (56).

The amino acid molar ratio called Fischer’s ratio (FR), which represents branched chain amino acids (BCAAs) [leucine, valine and isoleucine] / aromatic amino acids (AAAs) [phenylalanine and tyrosine] concentration ratio is important for assessing liver metabolism, hepatic functional reserve and the severity of liver dysfunction (57,58), while simplified Fisher ratio (BTR), which represents BCAAs [leucine, valine and isoleucine] / tyrosine concentration ratio, is proposed as a substitute for Fischer’s ratio as an index of hepatic damage and later reported that it reflects the progression of chronic liver disease (59,60).

This current study, Fisher ratio (FR) showed significant difference between the two studied groups and also in comparison to control group with the most decrease in NC rather than BA group, while simplified Fisher ratio (BTR) showed significant difference between the two studied groups with significant decrease in NC rather than BA group compared to control group but BA group did not show that significant decrease compared to control group.
The present findings seem to be consistent with earlier studies by Delgado Dominguez et al., (1998) and Al Mardini et al., (2006)(41,60) that showed reduction in the BCAA/AAA molar ratio in chronic liver disease, which indicated that the condition of liver was worsening.

Also Byrd et al., (1993) (10) revealed that the Fischer index was significantly decreased in the BA patients versus controls

Similarly with the current results Rutgers et al., (1987) (61) showed that plasma amino acid analysis and determination of the molar ratio may be useful in the differential diagnosis of hepatocellular and obstructive jaundice. A decrease in the molar ratio may reflect hepatocellular disease (27).

On the other hand, assessing hepatic functional reserve from the perspective of fluctuations in plasma free-amino acid concentrations can prove useful in different ways compared with investigations of the degree of hepatic fibrosis, hepatic blood flow and hepatocyte function. BTR correlates with each of the various liver function examinations. BTR offers a significant indicator of reserve liver function (59, 62) as decreasing in Fischer ratio and simplified fisher ratio (BTR) with increasing severity of hepatic damage (60).

In the case of poor nutritional status, BTR decreases in advance of decreases in serum albumin level. For that reason, determination of BTR enables prediction of changes in the serum albumin level one year later (63). These data indicate that amino acid imbalance, either low Fischer ratio or BTR, is a marker for progression of liver diseases, and that correcting this ratio may have therapeutic potential, not only for nutritional improvement, but also for complication of liver failure as hepatic encephalopathy, in patients with advanced liver diseases (58).

Regarding carnitine and acyl carnitines, In this study there was statistically significant increase in free carnitine and short chain acyl carnitine which are acetyl carnitine(C 2), propionyl carnitine (C3), butyryl carnitine (C4) in studied patients groups compared to control group.

The current study found that medium chain acyl carnitine had statistical significant increase in isovaleryl carnitine (C 5), glutaryl carnitine (C5DC), hexanoyl carnitine (C6) in studied patients groups compared to control; however there was none such significant increase in octanoyl carnitine(C8) and decanoyl carnitine (C10).

In the current study there was statistically significant increase in long chain acyl carnitine which are dodecanoyl carnitine (C12), tetradecanoyl carnitine (C14), hexacanoyl carnitine (C16) and octadecanoyl carnitine(C18) in both studied patients groups compared to control group.

The study showed statistical significance difference between BA group and NC rather than BA group regarding butyryl carnitine (C4) and octadecanoyl carnitine (C18) being more increase in non BA group.

These current results seem to be in parallel with previous studies by Wennberg et al., (1992) (63) who reported that patients with liver disease had significant increase concentrations of free and all carnitine compared with controls. Also, Lee et al., (2006) (65) found an elevation of free carnitine, C2-carnitine, and long-chain acyl carnitines in cholestatic patients.

And in line with our study, Selimoglu et al., (2001) (64) reported that plasma carnitine levels were significantly increased in children with BA. Similarly Zhao et al., (2014) (7) revealed in his study significant increase in short chain acyl carnitines in BA patient.

The main function of carnitine is to shuttle activated long-chain fatty acids (fatty acyl-coenzyme A (CoA)) from the cytosol to the mitochondria matrix for β-oxidation, and to remove from mitochondria short-chain, medium-chain and long-chain fatty acids that accumulate as a result of normal and abnormal metabolism, maintaining adequate cellular levels of free CoA(66).

So these results suggest that effect of decrease bile function as it has important role in the elimination of long chain acyl carnitines (67).

Because carnitine is synthesized by the liver, severe liver disease produces profound disturbances in whole-body carnitine metabolism. However, studies conducted to determine the carnitine status of liver disease patients yielded conflicting results. This in part may be due to the varying severity and etiology of liver disease in the patients studied (68, 69).

Possible causes of elevated carnitine concentrations in blood from patients with liver disease include increased biosynthesis, decreased hepatic clearance and increased release from tissues. Given the effects of severe liver disease on synthetic function (70), it is unlikely that excess quantities of carnitine would have been synthesized. Thus, elevated blood levels of carnitine probably arose from either impaired hepatic uptake and/or excessive release from peripheral and hepatic tissues. Muscle contains a significant quantity of carnitine for fatty acid transport (71) loss of muscle mass may promote carnitine release into the blood (70). However all this findings should to be studied to reveal further mechanisms that lead to such pathophysiological changes (7).

5. Conclusion:
This study demonstrates the possibility of metabolomics as non-invasive biomarkers for the early detection of BA and also provides new insight into pathophysiology of BA.

There is common metabolic pathway of BA and other causes of neonatal cholestasis as all however there is difference in metabolic pathway could be detected by difference in metabolomics result.

Fisher ratio, simplified fisher ratio, C4 and C18 could be used as metabolomics marker differentiating BA from non BA causes of NC.

**Corresponding Author:**
Name: Israa M Ismail (MB.BCh)
Work address: Yassin Abdel-Ghaffar St., National Liver Institute, Minoufia University, Shebin El-kom, Menoufia, Egypt.
Email: Israataher@ymail.com

**References**


52. Mata JM, Kershobinlich D, Villarreal E and Rojkind M. Serum free proline and free hydroxyproline in...