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# Urinary Organic Acids as Non-Invasive Biomarkers of Egyptian Pediatric Liver after Direct-Acting Antivirals

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## ABSTRACT

**Background:** There are significant global efforts being made to eradicate the hepatitis C virus (HCV). The outcome of HCV treatment has altered significantly as a result of the use of direct-acting antivirals (DAAs). The oral drugs sofosbuvir (SOF) and ledipasvir (LED) are recommended by the World Health Organization for the treatment of HCV in adolescents older than or equivalent to 12 years old. To the best of our knowledge, the impact of HCV clearance by DDAs on the metabolic pathways in the liver has not been examined in any metabolomics research to date.

**Objective:** This study was aimed to evaluate urinary organic acids before and after viral eradication by DDAs which reflect the metabolic status of the liver.

**Patients and Methods:** This prospective study included 50 pediatric cases of chronic hepatitis C (CHC) genotype 4 who fulfilled the inclusion and exclusion criteria in addition to 50 healthy controls. All participants underwent routine laboratory investigations, HCV polymerase chain-reaction (PCR) test and urinary organic acids using gas chromatography mass spectrometry (GC/MS) before and at the end of treatment.

**Results:** The study indicated that liver enzymes were decreased significantly after viral clearance (All $P < 0.05$), Urinary 2 keto-glutarate (P1<0.001), hippuric acid (P1<0.001), acetic acid (P1<0.001), phenylacetic acid (P1<0.002), palmitic acid(P1<0.007) and stearic acid (P1<0.027) were decreased significantly after treatment while L-fucose (P1<0.001), D-xylose (P1<0.001), arabinose (P1<0.002), L-rhamnose (P1<0.001), and D-ribose (P1<0.001) were increased significantly after treatment. Urinary organic acids profile after treatment became nearer to healthy control.

**Conclusion:** Viral clearance by DDAs has improved the metabolic functions of the liver so it is an effective treatment. Metabolomics can be used as non-invasive biomarker to assess the liver pathological and physiological state and to assess the efficacy of new drugs.

**Keyword:** Pediatric HCV, Direct Acting Antivirals, Urinary organic acids, Gas chromatography-mass spectrometry

## INTRODUCTION

More than 71 million people worldwide are plagued by chronic hepatitis C (CHC), which has a 1% global prevalence [1]. Cirrhosis and its related effects, including portal hypertension, liver decompensation, hepatocellular carcinoma (HCC), liver inflammation, and progressive fibrosis, are brought on by infection [2].

More than 5 million children globally have active HCV infection, despite the fact that most instances are in adults [3]. Pediatric patients in Egypt were shown to have a significant prevalence of HCV, ranging from 1.4 to 5.8% [4, 5]. Interferon and ribavirin were the recommended treatments for both adults and children over the age of 3; however, they have serious side effects and a sustained virological response (SVR) that does not exceed 63% [6]. Their antiviral activity involved the removal of infected hepatocytes over time via the immune system.

With extremely high cure rates (over 95%), short treatment times, excellent tolerability, and low treatment failure rates, direct-acting antiviral drugs (DAAs), which target proteins implicated in HCV replication, have altered the therapy landscape [7]. According to WHO guidelines, adolescents with chronic HCV who are 12 to 17 years old and weigh at least 35 kg but do not have cirrhosis or only have compensated cirrhosis should get SOF/LED for 12 weeks. It has been hypothesized that DAAs cause a swift improvement in liver homeostasis by causing a drop in intracellular viral burden that eventually results in its removal (cell cure) [8]. The liver is the main organ in which most metabolic processes, such as detoxification of blood, production of bile, glucose storage in the form of glycogen, and amino-acid precursor synthesis, take place. 85% of hepatic cells are responsible for these metabolic processes, So the metabolic shift which occurs in liver diseases is not surprising and it can be seen in metabolites in the blood and urine [9].

The aim of this work was to analyze the urinary organic acid profile of HCV-infected children, who received DAAs therapy and following them up till HCV clearance with comparing them with matched age and sex control group to assess the impact of HCV clearance.

## PATIENTS AND METHODS

This prospective study included a total of 50 pediatric patients infected with hepatitis C virus (Group 1) attending at National Liver Institute, Menoufia University. Fifty apparently healthy children of matching age and gender served as control (Group 2) were also recruited.

**Inclusion criteria:** All verified chronic HCV cases, regardless of treatment history or age (12–18 years or weight 35 kg).



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Exclusion criteria: Patients with HBV infection. Patients with cirrhosis. Post liver transplantation cases, cases with primary hematological disorder not related to chronic HCV infection, patients with estimated glomerular filtration rate eGFR< 30ml/min/1.73m$^2$, cases with cardiac disease and concomitant malignancy.

**Treatment regimen:**
For a period of 12 weeks, each kid received a fixed-dose SOF/LED (400 mg/90 mg), one tablet per day at a set time. Heterosofir Plus tablets, a generic medication, were utilised in our investigation.

**Workup before start of therapy:**
All cases had a complete clinical examination, a full history taking, abdominal ultrasonography, transient elastography (Fibroscan, Echosens, France) for assessing liver fibrosis, and a list of laboratory investigations including (1) Anti-HCV, HCV-RNA, and HCV genotype using an enzyme-linked immunosorbent assay (ELISA) of the fourth generation for hepatitis C virus antibodies (Innogenetics, Ghent-Belgium). (2) Hepatitis B virus serology: Hepatitis B surface antigen (HBsAg), anti-hepatitis B core IgM, and anti-hepatitis B core IgG types were assessed using an ELISA kit (Sorin Biomedica Co, Saluggia, Italy); (3) routine lab examinations using the Clinical Auto analyzer (Beckman Instruments, Fullerton, CA, USA) including liver function tests aspartate transaminase (AST), alanine transaminase (ALT), serum total and direct bilirubin, serum albumin (Alb), prothrombin time (PT), serum α-fetoprotein (AFP), serum creatinine, with estimated glomerular filtration rate (eGFR), and a test for urinary organic acids.

**Work up at the end of treatment:**
All cases had a thorough clinical examination and a battery of tests at the conclusion of the treatment, including tests for HCV-RNA, total and direct bilirubin, AST, ALT, serum albumin, serum creatinine, and urine organic acids. The HCV-RNA test was performed again after 3, 6, and 12 months, and all results were negative.

**Sample collection for urinary organic acids assay:**
Without using any preservatives or dietary restrictions, urine samples from all patients were collected aseptically and stored immediately at -80 °C until the organic acids were analyzed using gas chromatography/mass spectrometry (GC/MS).

**Ethical considerations**
This study was ethically approved by the National Liver Institute's ethics committee (IRB 00290/2022 INTM). Written informed consent of all the participants' parents was obtained. The study protocol conformed to the Helsinki Declaration, the ethical norm of the World Medical Association for human testing.

***Statistical analysis***
SPSS, version 26, was used to gather, tabulate, and statistically analyse the data (SPSS Inc, Chicago, IL, USA). Quantitative data were expressed as mean (x), standard deviation, and number (N), while qualitative data were expressed as percentage (%). (SD). Depending on the type of data, the Student's t-Test or non-parametric Mann-Whitney U test was used to determine the statistical significance of quantitative data. Depending on the type of data, the paired t test and Wilcoxon test were employed to compare the pre- and post-treatment data. The Chi-square test or Fisher's exact test was used to determine the importance of qualitative data. If the p value was less than 0.05, the results were deemed significant.

**RESULTS**
The mean age of presentation in CHC cases was 13.88±1.27 years, 54% male with mean weight 48.4 ± 15 kg, the mean viral load before treatment was 574.396±129.732 IU/ml. Every case that was analyzed had HCV genotype 4. All cases tested negative for PCR at the end of treatment, a 100% response rate. Only two cases (4%) exhibited a substantial degree of fibrosis, whereas the majority of patients (96%) showed none to mild fibrosis (F0/F1) as determined by fibro scan (F2).

Table 1 shows the demographic and clinical data of the studied groups. It shows no statistically significant differences among cases and control group, regarding age, sex, prothrombin time (PT) and AFP (All $P > 0.05$).

**Table (1): Comparison between cases and controls regarding demographic data, Alpha fetoprotein and PT.**

| Variable | Group1 No.=50 | Control No.=50 | Test ofsignificant | P value |
|---|---|---|---|---|
| **Age** (years)<br>Mean ±SD<br>Range | <br>13.88±1.27<br>(12 – 16) | <br>13.5±1.4<br>(11-16) | <br>t =1.419 | <br>0.159 |
| **Sex**<br>Male<br>Female | **No. %**<br>27(54.0)<br>23(46.0) | **No. %**<br>22(44.0)<br>28(56.0) | **$\chi$2=**0.485 | 0.486 |
| Alpha- fetoprotein (ng/ml) Mean ±SD | 2.23±0.53 | 1.98±0.45 | U=0.521 | 0.602 |
| PT (seconds)<br>Mean ±SD | <br>12.71±0.59 | <br>12.62±0.41 | <br>t =0.903 | <br>0.369 |

t = student t test. $\chi^2$= Chi-Square test, U= Mann-Whitney test. P value of < 0.05: statistically significant

Table 2 shows that ALT, AST, total bilirubin (T. Bil), direct bilirubin (D. Bil) were significantly decreased after treatment compared to pre-treatment (All $P < 0.05$). Also, there were no statistically significant differences in serum albumin and creatinine, also pre- treatment cases showed significantly increased ALT, AST, T.Bil, D.Bil and creatinine compared to control. Lastly ALT, AST, T.Bil, and creatinine were significantly increased in post treatment cases compared to control group.

**Table (2): Difference of liver and kidney function tests before and after treatment in studied patients and controls (n=50).**

| Variable | Pretreatment No.=50 Mean ±SD Range | Post treatment No.=50 Mean ±SD Range | ControlNo.=50 Mean ±SD Range | Test of significance | P value |
|---|---|---|---|---|---|
| ALT *(IU/L)* Mean ±SD | 33.96±8.11 | 21.6±5.14 | 11.7±2.75 | 5.685[a]<br>8.375[d]<br>7.799[d] | P1<0.001**<br>P2<0.001**<br>P3<0.001** |
| AST *(IU/L)* Mean ±SD | 33.98±8.22 | 21.32±4.75 | 17.28±3.46 | 4.962[a]<br>7.356[d]<br>4.857[c] | P1<0.001**<br>P2<0.001**<br>P3<0.001** |
| Total bilirubin (mg/dl) Mean ±SD | 0.65±0.15 | 0.56±0.11 | 0.36±0.08 | 4.296[b]<br>6.333[d]<br>6.191[d] | P1<0.001**<br>P2<0.001**<br>P3<0.001** |
| Direct bilirubin (mg/dl) Mean ±SD | 0.17±0.03 | 0.12±0.02 | 0.12±0.03 | 3.549[a]<br>3.5[d]<br>0.626[d] | P1<0.001**<br>P2<0.001**<br>P3=0.532 |
| Serum Albumin (g/dl) Mean ±SD | 4.58±0.43 | 4.64±0.44 | 4.56±0.48 | 1.566[b]<br>0.262[c]<br>0.892[c] | P1=0.124<br>P2=0.794<br>P3=0.375 |
| Creatinine (mg/dl) Mean ±SD | 0.64±0.11 | 0.66±0.13 | 0.54±0.12 | 1055[b]<br>3.820[c]<br>3.931[d] | P1=0.296<br>P2<0.001**<br>P3<0.001** |

AST: Aspartate transaminase, ALT: Alanine transaminase, **P value of < 0.001: statistically highly significant.
P1 **Comparing** between pretreatment and post treatment results, P2 **Comparing** between pretreatment and control, groups, P3 **Comparing** between post treatment and control groups.a = Wilcoxon test c= student t test, b = Paired t test d= Mann-Whitney test

**Urinary organic acids in the enrolled groups:**

Analysis of urinary organic acids of the studied groups are shown in table 3 and figure 1. There were statistically significant differences in pre-treatment cases compared to after treatment cases. There was significant increase of 2 keto-glutarate (P1<0.001), hippuric acid (P1<0.001), acetic acid (P1<0.001), phenylacetic acid (P1=0.002), palmitic acid (P1=0.007) and stearic acid(P1=0.027). While there was significant increase of L-fucose(P1=0.001), D-xylose(P1<0.001), Arabinose(P1=0.002), L-rhamnose(P1<0.001), D-tagatose(P1=0.001) and D-ribose(P1<0.001) in post treatment cases compared to pre-treatment cases.

**Table (3): Difference of organic acids values before and after treatment in studied patients and controls.**

| Organicacids | Pretreatment (NO.=50) | | Post treatment (NO.=50) | | Control (NO.=50) | | Test of significant | P value |
|---|---|---|---|---|---|---|---|---|
| | +ve | -ve | +ve | -ve | +ve | -ve | | |
| **Urea** | 48 | 2 | 49 | 1 | 21 | 29 | **$\chi^2$**=34.081<br>**$\chi^2$**=37.333 | P1=1 P2<0.001**<br>P3<0.001** |
| **Phosphate** | 32 | 18 | 31 | 19 | 15 | 35 | **$\chi^2$**=11.602<br>**$\chi^2$**=10.306 | P1=1 P2=0.001**<br>P3=0.001** |
| **2 keto-glutarate** | 22 | 28 | 3 | 47 | 3 | 47 | **$\chi^2$**=19.253 FE=0 | P1<0.001**<br>P2<0.001**P3=1 |
| **Tetra decanoicacid** | 5 | 45 | 0 | 50 | 1 | 49 | **FE=2.837**<br>**FE=1.010** | P1=0.063<br>P2=0.204<br>P3=1 |
| **Hippuricacid** | 20 | 30 | 4 | 46 | 3 | 47 | **$\chi^2$=16.318**<br>**FE=0.154** | P1<0.001**<br>P2<0.001**P3=1 |
| **3hydroxipropionic acid** | 4 | 46 | 1 | 49 | 3 | 47 | FE=0.154<br>FE=1.042 | P1=0.250<br>P2=1<br>P3=0.617 |

| Organicacids | Pretreatment (NO.=50) | | Post treatment (NO.=50) | | Control (NO.=50) | | Test of significant | P value |
|---|---|---|---|---|---|---|---|---|
| | +ve | -ve | +ve | -ve | +ve | -ve | | |
| **Citric acid** | 6 | 44 | 4 | 46 | 3 | 47 | FE=1.099<br>FE=0.154 | P1=0.687P2=0.295<br>P3=1 |
| **Glycerol** | 2 | 48 | 6 | 44 | 3 | 47 | FE=0.211<br>FE=1.099 | P1=0.289P2=0.646<br>P3=0.487 |
| **Vanillic acid** | 1 | 49 | 1 | 49 | 3 | 47 | FE=1.042<br>FE=1.042 | P1=1<br>P2=0.617P3=0.617 |
| **Lactic acid** | 3 | 47 | 1 | 49 | 0 | 50 | FE=3.093<br>FE=1.00 | P1=0.625<br>P2=0.242<br>P3=1 |
| **3hydroxi butyric acid** | 1 | 49 | 0 | 50 | 0 | 50 | FE=1.010 | P1=1<br>P2=1<br>-- |
| **Acetic acid** | 14 | 36 | 0 | 50 | 4 | 46 | **χ2**=6.775<br>FE=4.167 | P1<0.001**<br>P2=0.009*<br>P3=0.117 |
| **Phenyl acetic acid** | 11 | 39 | 1 | 49 | 3 | 47 | **χ2**=5.316<br>FE=1.042 | P1=0.002*<br>P2=0.021*P3=0.617 |
| **Succinic acid** | 2 | 48 | 6 | 44 | 3 | 47 | FE=0.211<br>FE=1.099 | P1=0.219<br>P2=1<br>P3=0.487 |
| **Aconiticacid** | 4 | 46 | 8 | 42 | | | FE=0<br>**χ2=1.515** | P1=0.344<br>P2=1<br>P3=0.357 |
| **Ascorbic acid** | 1 | 49 | 0 | 50 | 2 | 48 | FE=0.344<br>FE=2.041 | P1=1<br>P2=1<br>P3=0.495 |
| **Palmitic acid** | 45 | 5 | 34 | 16 | 22 | 28 | **χ2**=23.926<br>**χ2=5.844** | P1=0.007*<br>P2<0.001**<br>P3=0.026* |
| **Stearic acid** | 43 | 7 | 32 | 18 | 18 | 32 | **χ2**=26.272<br>**χ2=7.870** | P1=0.027*<br>P2<0.001**<br>P3=0.009* |
| **l-fucose** | 1 | 49 | 12 | 38 | 6 | 44 | FE=3.840<br>**χ2=2.439** | P1=0.001**<br>P2=0.112 P3=0.192 |
| **D-xylose** | 1 | 49 | 19 | 31 | 7 | 43 | FE=4.891<br>**χ2=7.484** | P1<0.001**<br>P2=0.027*<br>P3=0.011* |
| **Arabinose** | 0 | 50 | 10 | 40 | 5 | 45 | FE=5.263<br>**χ2=1.961** | P1=0.002*<br>P2=0.056P3=0.161 |
| **L-Rhamnose** | 0 | 50 | 16 | 34 | 5 | 45 | FE=5.263<br>**χ2=7.294** | P1<0.001**<br>P2=0.056<br>P3=0.007* |
| **D -tagatose** | 0 | 50 | 11 | 39 | 4 | 46 | FE=4.167<br>**χ2=3.843** | P1=0.001**<br>P2=0.117 P3=0.05 |
| **D-ribose** | 0 | 50 | 13 | 37 | 5 | 45 | FE=5.263<br>**χ2=4.336** | P1<0.001**<br>P2=0.056<br>P3=0.037* |
| **Fructose** | 0 | 50 | 2 | 48 | 4 | 46 | FE=4.167<br>FE=0.709 | P1=0.5 P2=0.117<br>P3=0.678 |
| **Ribitol** | 0 | 50 | 5 | 45 | 2 | 48 | FE=2.041<br>FE=1.382 | P1=0.063P2=0.495<br>P3=0.436 |

*P value of < 0.05: statistically significant. **P value of < 0.001: statistically highly significant. P1 between pretreatment and post treatment results, P2 between pretreatment and control groups, P3 between post treatment and control groups.
**χ2**= Chi-Square test, FE= Fisher's Exact test.

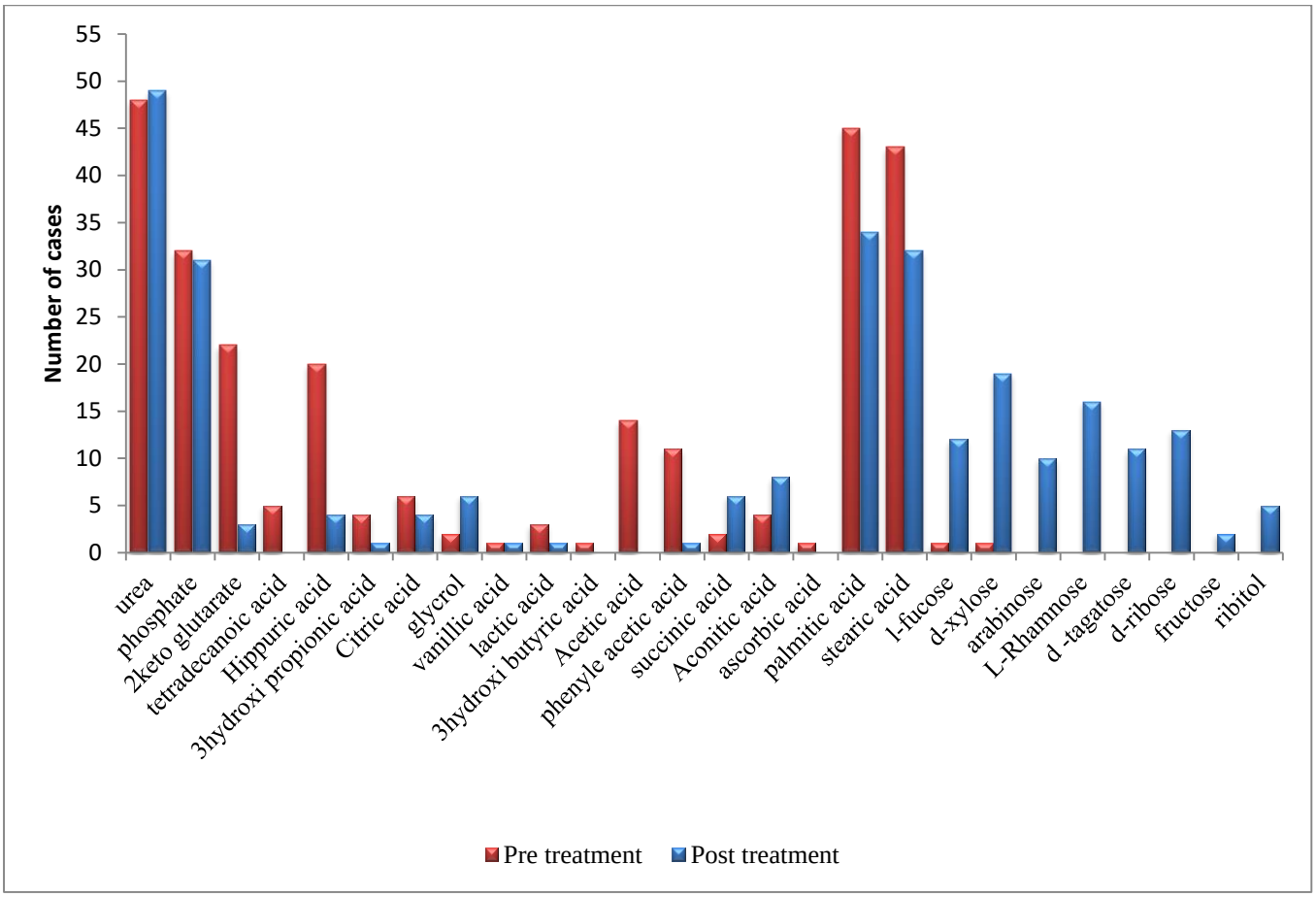


**Figure (1): levels of organic acids values before and after treatment in studied patients (positive results).**

## DISCUSSION

Few metabolomic investigations in paediatric HCV-infected individuals have been carried out. [10]To the best of our knowledge, this prospective longitudinal study was the first to examine the changes in urine organic acids in juvenile HCV patients before beginning DAAs treatment and after finishing treatment using GC-MS metabolomics.[10] This study involved 50 individuals who had been diagnosed with chronic HCV through the presence of positive HCV-RNA. Additionally, 50 seemingly healthy patients who were age and gender matched acted as a control group. At the National Liver Institute's Pediatric Hepatology, Gastroenterology, and Nutrition Department, patients were looking for medical advice.

Comparing PCR before and at the end of treatment, 100% response rate the end of treatment EOT was found. PCR was repeated after 3, 6 and 12 months and it was found to be remained negative for all cases. This was in agreement with several studies **Behairy *et al.* [11], El-Karaksy *et al.* [12] and Yakoot *et al.* [13]** who reported rapid SVR in children which reached up to 97% during treatment with DDAs treatment.

ALT, AST, T.Bil, and direct bilirubin were all shown to be significantly lower following therapy when compared to pre-treatment cases, treatment outcomes, and healthy control group. These results were in line with those of **Yakoot *et al.* [13],** who stated that at the end of the study, all patients had normalized liver enzymes along with normal hematological, liver, and renal function tests. According to **Behairy et al. [11]**, ALT and AST levels reverted to normal starting in the second week of treatment when compared to pre-treatment levels. Since these are the intracellular hepatic enzymes that have entered the bloodstream as a result of liver cell damage and act as indicators of hepatocyte damage. They were discovered to be lower after treatment because the hepatocytic injury caused by the HCV was resolved, which also explains why total and direct bilirubin levels were lower after treatment.

Urinary organic acids showed significant increase of (2 keto-glutarate, Hippuric acid, Acetic acid, phenylacetic acid, palmitic acid and stearic acid) in pretreatment cases compared to post treatment cases. And there was significant increase of (Urea, Phosphate, 2 keto-glutarate, hippuric acid, acetic acid, phenylacetic acid, palmitic acid and stearic acid) in pretreatment cases compared to control. This was in accordance with **Meoni *et al.*[14]** who found that 2 keto-glutarate and Acetate were decreased in HCV patients after DDA treatment. He attributed changes to multiple processes, including lysine biosynthesis (2-oxoglutarate), pyruvate metabolism, and acetate metabolism for the rise in 2 keto-glutarate and acetate levels (lactate, formate, acetate, pyruvate).

Our findings about elevated levels of palmitic and stearic acids in the urine in cases prior to treatment were consistent with those of **Arain et al. [15],** who demonstrated that increasing levels of unsaturated fatty acid levels were positively associated with decreasing

levels of hepatitis C virus replication. The serum free fatty acids (FFA) profile of acute HCV patients revealed significantly higher levels of palmitic and stearic acid in pre-treated patients, which were then decreased after treatment.

This can be explained by the fact that fatty acid synthase (FAS), an enzyme primarily engaged in the de novo synthesis of fatty acids, is increased during HCV infection to provide essential lipid components for the development of successful HCV replication [15].

Our study found that there was a significant increase of (L-fucose, D-xylose, Arabinose, L-rhamnose, D-tagatose and D-ribose) in post treatment cases compared to pretreatment. This was in agreement with **Osman *et al.*** [65] who revealed that arabinose and xylitol were significantly increased in healthy control compared to liver cirrhosis patients.

## CONCLUSION

DDAs in HCV pediatric population is an effective and safe therapy to use that leaded to 100% response rate with normalization of liver enzymes and urinary metabolomics and this of course reflect the improvement of liver cell injury and overall liver conditions.

Metabolomics can serve as simple non-invasive tool for assessment of pathophysiological state of liver and response to treatment.

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