Adequate Chelation and Cupriuresis in Hepatic Wilson disease patients under Combination (Chelator + Zinc) therapy at 2 years of follow up

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Research Article

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Abstract

Purpose

Role of 24-hour urinary copper excretion (UCE) in treatment monitoring of Wilson disease (WD) is not well studied especially in pediatric population. Hence, present study is conducted with aim to evaluate UCE and its role in deciding therapeutic adequacy in paediatric WD on long-term follow-up.

Methods

All WD patients < 18 years and on combination therapy with atleast one UCE available after first year of treatment were included. Liver biochemistries, UCE (mcg/day) and serum non-ceruloplasmin bound copper (NCC) (mcg/dl) were assessed at diagnosis and various follow-ups. For assessment of treatment efficacy, criteria for adequate chelation (CAC) was defined as fulfilment of both (i) AST & ALT ≤ 1.5 times upper limit of normal, serum albumin > 3.5 gm/dl, INR < 1.5 and (ii) UCE < 500.

Results

Of the 74 included children, 70 (94.5%), 45 (60.8%), 28 (37.8%) and 21 (28.3%) completed 2-, 3-, 5- and 7-years follow-up respectively. Liver biochemistries improved significantly within 1 year of treatment. UCE decreased significantly from baseline of 654.08 ± 803.78 to 308.23 ± 175.93 at 2 years with no further change at 3 & 5 years follow-up. UCE at 2 years was < 200 in 28.5%, 200–500 in 55.7%, and > 500 in 15.7%. 61% achieved CAC by 2 years. On multivariate cox regression, treatment compliance was predictor for CAC achievement (p = 0.009, HR: 3.48, 95% CI: 1.36–8.86).

Conclusion

UCE declines significantly from baseline to < 500 mcg/day within 2 years. Majority of treatment compliant patients achieve CAC within 2 years of combination therapy.

What Is Known

• 24 hours urinary copper excretion (UCE) is one of the recommended biochemical diagnostic tools for Wilson disease.
• UCE might be helpful in assessment of therapeutic efficacy during follow-up.

What Is New

• Criteria for adequate chelation (CAC) is formulated and could be a valuable guide for monitoring treatment adequacy for hepatic WD patients.
• 80% of treatment compliant patients achieve CAC with 2 years of combination (Chelator + Zinc) therapy. Treatment compliance is a significant predictor for achievement of CAC

**Introduction**

Wilson's disease (WD) is a genetic disorder with progressive accumulation of copper in the liver, brain, corneas, kidneys, and heart [1-4]. The presentation in children and younger adults range from a milder phenotype to compensated/decompensated cirrhosis with portal hypertension or acute liver failure/acute-on-chronic liver failure [5-7]. Apart from diagnosis [5-7], 24-hour urine copper excretion (UCE) plays a vital role in the assessment of therapeutic efficacy during follow-up period. UCE usually remains in the range of 200-500 mcg/day after 1-2 years of initiation of chelation therapy in the well-controlled compliant patients [8-9]. Till date, only one paediatric study has been published which suggests that there occurs a significant reduction in UCE after 1-2 years of treatment as compared to the pre-treatment values [10]. Furthermore, there is no published literature on the follow-up UCE values of the WD patients receiving combination therapy of a chelator and Zinc (Zn). We can hypothesize that UCE values will be lower and normalise earlier in those on combination therapy. Hence, this present study was conducted with a primary objective to evaluate UCE rates in pediatric WD patients receiving combination therapy (Chelator + Zn) at different time points on long-term follow-up.

**Methods**

We have evaluated prospectively collected clinical and laboratory data of all patients less than 18 years of age diagnosed as WD at our institute from 2014 to 2021. The study was approved by Institutional Review Board and Institutional Ethics Committee (IEC approval no: IEC/2020/76/NA05). The diagnosis of WD was made as per the European Association for the Study of the Liver (EASL) guidelines, where a Leipzig score of 4 or more was considered diagnostic [5-6]. At our institute, genetic analysis of all the suspected WD patients is conducted for five common mutations in ATP7B gene (3182 G > A, 813 C > A, 1708-1 G>C, 3305 T>C and 448-452 deletion) prevalent in northern India.

Only those patients who were receiving combination therapy (a chelating agent either D- Penicillamine (D-Pen) / Trientine along with Zn) and had undergone UCE analysis once after first year of initiation of treatment were included in the study. WD patients on Zn monotherapy or combination treatment for less than one year were excluded from the study. Both the drugs were given in two divided doses, 6 hours apart from each other and two hours away from meals. Trientine was added to those who could not tolerate D-Pen. All of our WD patients were prescribed Zn-acetate and dosing schedule was as per the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition position paper on WD published in 2018 [7].

UCE and Non ceruloplasmin bound copper (NCC) values were assessed at the start of therapy as well as at 1-, 2-, 3-, 5-, and 7 years after initiation of combination therapy. NCC was calculated by using the equation: NCC = (Serum total copper – 3 x Serum Ceruloplasmin). UCE and serum copper analysis were performed using inductively coupled plasma mass spectrometry (ICP-MS) [11-12]. Acid washed plastic container was
used for collection of urine samples for UCE estimation [7]. As per our institutional protocol, neither D-Pen challenge (before baseline UCE estimation at diagnosis) nor D-Pen dose interruption (prior to follow up UCE analysis) were done for the patients. Apart from copper studies, data regarding compliance, liver biochemical tests, international normalized ratio (INR), haemogram were also recorded at each follow-up time point to assess the disease control status. Poor compliance (both missed and late doses) was defined as a patient taking less than 80% of the prescribed doses of D-Pen or Zn or both [13]. For comparison of laboratory profiles of the compliant patients versus those with poor compliance the data from the defaulter phase was collected. We have developed a composite end point including liver biochemistries and copper studies to identify the proportions of the patients achieving adequate chelation on combination therapy at various time points in the follow up. It was designated as “Criteria for Adequate Chelation” (CAC) and defined as the patient fulfilling all the following four criteria: (i) aspartate aminotransferase (AST) and alanine aminotransferase (ALT) within 1.5 time of upper limit of normal (ULN), (ii) INR < 1.5, (iii) serum albumin > 3.5 gm/dl, (iv) UCE < 500 mcg/day. ULN for AST and ALT was kept as 40 IU/L in our study as per the international consensus [14]. For the multivariate analysis we calculated the average dose of the chelator in the subjects in mg/kg/day. New Wilson Index (NWI), a WD prognostic score [15] was calculated for all the enrolled children at index presentation.

Statistical analysis: SPSS version 28 (IBM SPSS Statistics, IBM Corporation, Armonk, NY) statistical package was used for statistical analysis. All quantitative variables (expressed as mean ± standard deviation/median and interquartile range (IQR) as appropriate) were analysed by repeated measures analysis of variance (ANOVA) test to compare mean values in the follow up visits. Independent sample ‘t’ test was used for comparison of the follow up liver function parameters and copper studies between patients with good compliance versus poor compliance. The qualitative variables (expressed as proportions) were analysed with Chi-Square/Fisher Exact test as appropriate. Non-parametric test was used for comparison of the data showing skewed distribution. Cox regression analysis was used to identify predictive factors affecting the achievement of CAC among the patients under combination therapy. A p value of < 0.05 is considered statistically significant.

Results

During the 7 years of study period, a total of 135 pediatric patients were diagnosed as WD. Of these 23 patients died, 17 have been transplanted and follow up data of 6 patients was not complete. Of the 89 patients surviving with native liver with complete follow up data, 15 patients were excluded from the study due to following reasons (8: only on Zn monotherapy, 7: less than 1 year since initiation of treatment). Hence, total 74 patients (38 genetically proven and 15 with additional neurological involvement) were included in the study. The median age of the cohort was 9.7 years (IQR: 8.2-12.0) at diagnosis and median follow up period was 3 years (IQR: 2-6 years). Among these 74 patients, 42 (56.8%) had presented as decompensated chronic liver disease and 7 (9.5%) fulfilled the acute-on-chronic liver failure definition of Asia Pacific association of study of liver disease [16] at presentation. Twenty-five (33.8%) patients had an initial manifestation of compensated chronic liver disease (hepatomegaly/ elevated transaminases). Thirty-eight (51.3%) of the enrolled children tested positive for one or more of the five common WD mutations
(813C>A (n=14) and 3182G>A (n=14) being the two most commonly detected mutations in our cohort. Of the total 74 cases, 20 (27%) showed evidence of coomb’s negative hemolysis at index presentation. Poor compliance to treatment was reported by 19 (25.6%) patients. Of the 74 included subjects, 70 (94.5%), 45 (60.8%), 28 (37.8%) and 21(28.3%) children had completed 2, 3, 5 and 7 years follow up respectively on combination therapy.

**Repeated Measures of the laboratory profile at various follow up visits:** The repeated measures of the laboratory profile of the cohort at the time of diagnosis (baseline) and subsequent follow-up are presented in table 1. AST, ALT, total bilirubin, albumin and INR improved significantly within 1 year of initiation of combination therapy (Table 1 & Figure 1). At index presentation, the mean AST and ALT (IU/L) were 118.71 ± 70.92 and 72.10 ± 68.93, which by 1 year declined to 49.62 ± 21.27 (mean difference: 69.09 (95% CI: 49.05-89.12), p value: < 0.001) & 50.12 ± 37.30 (mean difference: 22.97, 95% CI: 3.80-40.15, p value: 0.012) respectively. All liver biochemistries except ALT & total bilirubin showed a further statistically significant improvement at 2 year follow up. The baseline albumin (g/dl) and INR of 2.69 ± 0.84 & 1.95 ± 0.70 completely normalized to 4.14 ± 0.41 (mean difference: 1.44, 95% CI: 1.20-1.69, p value: < 0.001) & 1.21 ± 0.16 (mean difference: 0.73, 95% CI: 0.52 - 0.94, p= < 0.001) respectively after 2 year of combination treatment. These improved liver parameters were maintained and we did not notice any further significant change in liver enzymes on 3 year and 5 year follow up (Table 2). However, liver enzymes of poorly compliant patients did not normalize and remained significantly high compared to compliant patients on follow up (Table 3). A statistically significant decrease in total leukocyte count (thousands/mm$^3$) [baseline: 7.52 ± 3.90 versus 1 year: 5.73 ± 2.34, mean difference: 1.78, 95% CI: 0.68 -2.88, p = < 0.001] was noticed after 1 year of combination treatment possibly because of the D-Pen related bone marrow suppression. However, platelet count did not show any significant change.

**24-hour urine copper excretion and Non-ceruloplasmin bound copper on follow-up:** UCE (mcg/day) declined steadily after introduction of combination treatment from a baseline value (mean ± SD) of 654.08 ± 803.78 to 412.97 ± 315.23 at 1 year (mean difference: 241.10, 95%CI: 4.89 - 487.10, p= 0.057) and 308.23 ± 175.93 at 2 year (mean difference: 345.85, 95%CI: 111.29 - 580.41, p= 0.002). Although, a statistically significance improvement in UCE was reached only at 2 year post combination therapy compared to baseline, we could notice a statistically significant decrease in UCE on comparison between 1 year and 2 year post combination treatment follow-up (mean difference:104.74, 95% CI: 11.46 – 198.02). However, no further significant change in the UCE values were observed at 3- and 5- years follow up compared to 2 years follow-up values (Table 2). Despite a decline in UCE values from baseline 655.70 ± 774.80 mcg/day to 5- years follow up value of 336.92 ± 337.91 mcg/day, it could not reach statistical significance which might be because of a smaller sample size (n=28). UCE at 1 year was less than 200 mcg/day in 15/74 (20.2%), 200-500 mcg/day in 43/74 (58.1%) and more than 500 mcg/day in 16/74 (21.6%). UCE at 2 years was less than 200 mcg/day in 20/70 (28.5%), 200-500 mcg/day in 39/70 (55.7%) and more than 500 mcg/day in 11/70 (15.7%). Furthermore, follow up UCE in non-compliant subjects (560.66 ± 323.62 mcg/day) was significantly higher in comparison to the compliant subjects (281.26 ± 160.04 mcg/day) (mean difference: 279.40, 95% CI: 163.68-395.13, p= < 0.001) (Table 3).
NCC (mcg/dl) also showed a similar trend with significant improvement from baseline value of 34.57 ± 17.37 to 1 year 2.69 ± 14.58 (mean difference: 31.87, 95% CI: 24.72 - 39.02, p=<0.001) and 2 year 2.61 ± 11.20 follow up (mean difference:31.95, 95% CI: 25.71 - 38.19, p=< 0.001). No significant change was found in 3- and 5- years follow-up as compared to 2- years follow up. Among the 70 cases, 52 (74.2%), 11(15.7%) and 7(10.0%) had NCC < 5, 5-15 and >15 mcg/dl respectively at 2- years follow-up. A significantly lower NCC was observed in patients with good compliance (1.05±10.49 mcg/dl) in contrast to those with poor compliance (6.33 ± 5.80 mcg/dl) on follow up (mean difference: -5.27, 95% CI: -0.19, -10.36, p=0.042) (Table 3). On comparison of NCC against the UCE values, it was found that NCC was comparable between those with UCE less than 200 mcg/day, 200-500 mcg/day and more than 500 mcg/day by one way ANOVA at 1- and 2- years follow-up. Further among 52 cases with NCC <5 at 2 year follow up, 16 (30.7%) had UCE less than 200mcg/day, 27 (51.9%) had UCE 200-500 mcg/day and 9 (17.3%) had UCE >500 mcg/day.NCC was comparable between the sub groups of patients with UCE < 200 mcg/day, 200-500 mcg/day and > 500 mcg/day by one way ANOVA at 1- & 2- years follow ups.

Achievement of Criteria for Adequate Chelation: At 2 year follow up, 43/70 (61.4%) of the included subjects were able to achieve the CAC (Figure 2). Five more patients achieved CAC till 5 year follow up. Of the 74, 22 patients have still not attained CAC and are attending the follow up visits. However, on comparison between the good versus poor compliant patients, 43/55 (78.1%) with good compliance had achieved the CAC compared to only 5/19 (26.3%) with poor compliance (OR: 1.98, 95%CI 1.29-3.03, p=0.000) (figure 2). Additionally, we also found that a significantly larger proportion of the children with 2 year follow up UCE ≤ 500 mcg/day [43/59 (72.8%)] were able to achieve CAC during the 5 year follow up period in comparison to patients with 2 year follow up UCE >500mcg/day [3/11 (18.1%)] (OR:7.16, 95%CI: 1.68-30.42, p=0.006). We conducted a multivariate Cox regression analysis to identify the predictors influencing the achievement of CAC including five parameters such as index hepatic manifestation (decompensated/acute on chronic liver failure versus compensated chronic liver disease), NWI at presentation, presence/absence of Coombs negative hemolytic anemia at presentation, average chelation dose (mg/kg/day) and compliance to treatment. On multivariate analysis, treatment compliance was found to be the independent predictor affecting the achievement of CAC, (p=0.009, HR: 3.48, 95% CI: 1.36-8.86). Among the 59 patients with 2 year follow up UCE < 500 mcg/day, a significantly higher percentage [86.9% (n=40/46)] of good compliance group patients were able to achieve CAC as opposed to [23.0% (3/13)] among the poorly compliant group (OR: 2.48, 95% CI: 1.31-4.69, p= < 0.001). On selecting patients who achieved CAC (excluding UCE < 500 mcg/day) at 2 years, 3 years and 5 years, UCE values of these patients showed significant decrease between baseline to 2 years with no further significant decline (Table 4). The 95% CI of the UCE at 2 years in those who achieved CAC (excluding UCE < 500 mcg/day) was 244-331 mcg/day. The reasons for poor compliance shared by the patients were school timing hampering drug dosage, false sense of treatment completion and behavioral issues during adolescence.

Discussion

To the best of our knowledge, our work is the first one to evaluate the long term follow up UCE for the hepatic WD patients on combination (chelator + Zn) therapy. In the present study, we found that UCE
decreases significantly by 2 years of combination therapy to < 500 mcg/day. A similar trend was also observed in improvement of NCC from baseline to 2 year follow up. All the liver biochemistries tend to normalise within 1 year of combination therapy with proper treatment compliance. Sixty one percent of the patients in the present cohort attained the CAC by second year of treatment. However, we found that therapeutic compliance has a very strong role in achieving CAC as the percentage of patients fulfilling the CAC was approximately 3 times more in the good compliance group compared to the poor compliance group (78% versus 26%).

In our cohort, the baseline mean UCE was 654.08 ± 803.78 mcg/day (Median (IQR): 332.0 (195.70-788.19) without any D-Pen challenge which is comparable with the values reported from other hepatic WD cohorts [8, 10]. The observed decline in UCE at 2 year follow up compared to the baseline value was also in agreement with the findings of the previous published literature [8, 10]. Likewise, in a study from a German centre done among 321 WD patients [9], the author found significant decrease in UCE (performed after 48-hour D-Pen dose interruption) at 1 and 2 year follow up visits. However, the 2 year follow up UCE rates were higher in the studies by Pfeiffenberger et al [9] and Chanpong et al [10] as compared to our cohort. Their 2 year UCE values (mcg/day) were [median: 507.5 (range: 277.2 -2290.2)] and [median: 587 (IQR: 217.8-831.6)] respectively, whereas in our study it was 307.5 (169.0-447.7) mcg/day. In another study the 2 year follow up UCE was 223.5mcg/day in the hepatic WD cases [8] but the authors have not analysed the statistical significance of the reduction in UCE. Moreover, the significant decrease in the UCE levels in the pediatric study [10] was seen at 3-4 years follow up as contrast to 2 years in the present study. Apart from small sample size of the cohort in the study [10], another plausible explanation for this difference in UCE between their cohorts and our cohort might be attributed to the better and prompt treatment response to the combination therapy used in our cohort as against the chelator monotherapy used by the previous mentioned studies.

In the present study, among the 84% (59/70) patients with 2 year UCE < 500 mcg/day, 28.5% (20/70) had UCE even less than 200 mcg/day suggestive of lower UCE levels on combination therapy due to Zinc as against chelator monotherapy. Interestingly, whatever change we observed in UCE, it occurred within 2 year of treatment and after that there was no significant change at 3 year and 5 year follow up and it remained in the range of [median (IQR)]: 301.0 (151.0 – 426.0) at 3 year and 294.0 (74.1-442.5) at 5 year. In the previous pediatric study [10] the UCE at 3 & 5 year follow up visits was 145.2 and 369.6 mcg/day which is also within normal limits. The same was seen in another study when the UCE was done after 48 hour D-Pen dose interruption [9].

Similarly, NCC also decreased drastically from baseline value of 34.57 ± 17.37 to 2.61 ± 11.20 at 2 year follow up and the improvement was maintained at 3 year & 5 year follow up without any significant change. Among the cohort participants, 52/70 (734.2%) & 11/70 (15.7%) had their 2 year follow up NCC < 5 and 5-15 mcg/dl. These findings of ours were consistent with that of Chanpong et al [10]. They reported that 77.8% of their subjects had NCC < 5 mcg/dl (0.8 µmol/dl) and 2 year follow up NCC was [median (IQR): -5.04 (-13.86-4.41)] similar to our values [median (IQR): 0.57 (-1.6-5.22)]. However, we could not get any significant difference in NCC values in patients with UCE < 200, 200-500 or > 500 mcg/day at 2 year follow up. This limits the clinical utility of NCC as a tool to assess the adequacy of chelation although it has been
stated in the guidelines that a NCC value of < 5 mcg/dl is suggestive of over treatment. Indeed, among the 52 participants of our cohort with NCC < 5 mcg/dl, only 16/52 (30.7%) had UCE < 200 mcg/day a pointer towards over chelation, rest had UCE either between 200-500 mcg/day [n=27/52 (51.9%)] or even > 500 mcg/day [n=9/52 (17.3%)]. Hence, NCC is not a very good biochemical marker to assess the treatment efficacy/adequacy. This discrepancy could be due to overestimation of ceruloplasmin by immunological methods used in the majority of the laboratories resulting in false low levels of NCC [6].

Despite UCE being well known to play an important role in therapeutic monitoring in WD, its potential role in defining the adequacy of chelation has not been explored fully yet. Also, there is lack of a well-defined set of biochemical criteria for identification of patients who have achieved adequate chelation with treatment. Hence, we applied the CAC on the patients at 2 year follow up when significant improvement in the biochemical profile was observed. We found 61% of the present cohort had achieved CAC by 2 years, which is another strong pointer towards consideration of stopping chelation and continuing the Zn maintenance therapy in this set of hepatic WD cases. Moreover, compliance to treatment had major bearing on obtainment of CAC. As was reported 26% of the poorly compliant patients achieved CAC as against 78% with good compliance. UCE and NCC were significantly higher during poor compliant period in the subgroup versus those with good compliance. Rigorous counselling sessions on follow up visits can alter the poor compliance behaviour to treatment.

The results of the present study suggest that 80% of the patients with hepatic WD with adequate compliance to combination therapy can attain CAC by 2 years. On analysing those who achieved CAC (without UCE< 500mcg/day), we found the 95%CI of UCE was lying between 244 to 331 mcg/day by 2 years. This further suggests that the chelation is adequate by 2 years. Hence, we propose that the components of the CAC can be further validated for switching from combination therapy to Zn monotherapy. Limitations of this study is single centre study with relatively small sample size on subsequent follow up visits. However, among the pediatric studies of hepatic WD, this is the largest cohort reported to date. Moreover, this is the only scientific data available regarding the pattern of cupriuresis in hepatic WD patients on combination (chelator + Zn) therapy.

We conclude that UCE decreases significantly from baseline to <500mcg/day and majority of the patients, if treatment compliant, achieve CAC after 2 years of combination therapy. Approximately in one third of patients, it even declines to below 200 mcg/day which might increase the risk of copper deficiency. This group of patients should be considered for switching to Zn monotherapy. NCC is comparable between various grades of cupriuresis thus limiting its clinical utility as a biomarker for assessment of therapeutic adequacy. In this context, CAC used in the present study could be a valuable future guide for monitoring of treatment adequacy as well as for deciding the long-term therapeutic plans for pediatric hepatic WD patients, especially to select the group of patients who could be gradually switched over to Zn monotherapy to avoid the potential harmful side effects of D-Pen in the long run.

Abbreviations

ALT
Alanine aminotransferase
ANOVA
Analysis of variance
AST
Aspartate aminotransferase
CAC
Criteria for Adequate Chelation
D-pen
D- Penicillamine
EASL
European Association for the Study of the Liver
ICP-MS
Inductively coupled plasma mass spectrometry
INR
International normalized ratio
IQR
Interquartile range
NCC
Non ceruloplasmin bound copper
NWI
New Wilson Index
UCE
24-hour urine copper excretion
ULN
Upper limit of normal
WD
Wilson's disease
Zn
Zinc

**Declarations**

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**Conflicts of Interest/ Competing Interests:** All the authors declare no financial or non-financial conflict of interest.

**Authors’ contribution:** All authors contributed to the study conception and design. Data collection was done by Kalpana Panda. Analysis of results and interpretation was done by Seema Alam, Bikrant Bihari Ial,
Vikrant Sood, and Kalpana Panda. The first draft of the manuscript was written by Seema Alam and Kalpana Panda. All authors contributed to the critical revision of final manuscript for important intellectual content, approved the final version, and agreed to be accountable for all aspects of work. Seema Alam is the corresponding author of this manuscript and takes the responsibility of coordinating the work from its inception to publication and can be approached for access to raw data.

Ethics Approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Institutional ethics committee of Institute of Liver and Biliary Sciences, New Delhi vide letter no: IEC/2020/76/NA05

Consent to participate: Informed consent was obtained from parents of all children included in the study.

Consent for publication: NA

References


Tables

Table 1: Repeated Measures ANOVA for comparison of lab parameters between Baseline, 1 year and 2 year post Combination therapy (N=70)
<table>
<thead>
<tr>
<th>Variables</th>
<th>Time</th>
<th>Mean Difference (95% CI)</th>
<th>p Value (Between Pairs)</th>
<th>F statistics, p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AST (IU/L)</strong></td>
<td>At Diagnosis/Follow-up</td>
<td>Follow-up (Mean ± SD)</td>
<td></td>
<td></td>
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<tr>
<td>Baseline 118.71±70.92</td>
<td>1 year 49.62±21.27</td>
<td>69.09 (49.05, 89.12)</td>
<td>&lt;0.001</td>
<td>74.11 (&lt;0.001)</td>
</tr>
<tr>
<td>1 year 49.62±21.27</td>
<td>2 year 41.47±12.68</td>
<td>8.15 (2.14, 14.16)</td>
<td>0.004</td>
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<td>Baseline 118.71±70.92</td>
<td>2 year 41.47±12.68</td>
<td>77.24 (56.26, 98.22)</td>
<td>&lt; 0.001</td>
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<tr>
<td><strong>ALT (IU/L)</strong></td>
<td>Baseline 72.10±68.93</td>
<td>1 year 50.12±37.30</td>
<td>21.97 (3.80, 40.15)</td>
<td>0.012</td>
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<tr>
<td>1 year 50.12±37.30</td>
<td>2 year 42.47±19.85</td>
<td>7.65 (-2.09, 17.39)</td>
<td>0.174</td>
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<tr>
<td>Baseline 72.10±68.93</td>
<td>2 year 42.47±19.85</td>
<td>29.63 (10.23, 49.02)</td>
<td>0.001</td>
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<tr>
<td><strong>TBIL (mg/dl)</strong></td>
<td>Baseline 3.77±5.70</td>
<td>1 year 0.81±0.45</td>
<td>2.96 (1.28, 4.65)</td>
<td>&lt;0.001</td>
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<tr>
<td>1 year 0.81±0.45</td>
<td>2 year 0.84±0.52</td>
<td>-0.03 (-0.14, 0.07)</td>
<td>1.000</td>
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<tr>
<td>Baseline 3.77±5.70</td>
<td>2 year 0.84±0.52</td>
<td>2.93 (1.26, 4.60)</td>
<td>&lt;0.001</td>
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<td><strong>Albumin (gm/dl)</strong></td>
<td>Baseline 2.69±0.84</td>
<td>1 year 3.89±0.49</td>
<td>-1.19 (-1.45, -0.94)</td>
<td>&lt;0.001</td>
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<tr>
<td>1 year 3.89±0.49</td>
<td>2 year 4.14±0.41</td>
<td>-0.24 (-0.36, -0.13)</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>Baseline 2.69±0.84</td>
<td>2 year 4.14±0.41</td>
<td>-1.44 (-1.69, -1.20)</td>
<td>&lt;0.001</td>
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<td><strong>INR</strong></td>
<td>Baseline 1.95±0.70</td>
<td>1 year 1.27±0.17</td>
<td>0.67 (0.48, 0.87)</td>
<td>&lt;0.001</td>
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<td>1 year 1.27±0.17</td>
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<td>0.05 (0.01, 0.09)</td>
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<td>Baseline 1.95±0.70</td>
<td>2 year 1.21±0.16</td>
<td>0.73 (0.52, 0.94)</td>
<td>&lt; 0.001</td>
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<td><strong>UCE (mcg/day)</strong></td>
<td>Baseline 654.08±803.78</td>
<td>1 year 412.97±315.23</td>
<td>241.10 (-4.89, 487.10)</td>
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<td>2 year 412.97±315.23</td>
<td>0.022</td>
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<tr>
<td>Parameter</td>
<td>Baseline</td>
<td>1 year</td>
<td>2 year</td>
<td>3 year</td>
</tr>
<tr>
<td>-------------------------</td>
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<td>----------------</td>
</tr>
<tr>
<td><strong>NCC (mcg/dl)</strong></td>
<td>Baseline</td>
<td>34.57 ± 17.37</td>
<td>2.69 ± 14.58</td>
<td>2.61 ± 11.20</td>
</tr>
<tr>
<td></td>
<td>1 year</td>
<td>31.87 (24.72, 39.02)</td>
<td>0.08 (-3.87, 4.03)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 year</td>
<td>31.95 (25.71, 38.19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hemoglobin (gm/dl)</strong></td>
<td>Baseline</td>
<td>10.21 ± 1.79</td>
<td>11.94 ± 1.32</td>
<td>12.24 ± 1.38</td>
</tr>
<tr>
<td></td>
<td>1 year</td>
<td>-1.72 (-2.29, -1.15)</td>
<td>-0.30 (-0.65, 0.05)</td>
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</tr>
<tr>
<td></td>
<td>2 year</td>
<td>-2.02 (-2.63, -1.41)</td>
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</tr>
<tr>
<td><strong>Total Platelets count (Thousands/mm³)</strong></td>
<td>Baseline</td>
<td>127.96 ± 75.31</td>
<td>139.30 ± 81.87</td>
<td>146.96 ± 84.58</td>
</tr>
<tr>
<td></td>
<td>1 year</td>
<td>-11.34 (-27.88, 5.20)</td>
<td>-7.65 (-21.52, 6.20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 year</td>
<td>-19.00 (-39.37, 1.37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total Leukocyte count (Thousands/mm³)</strong></td>
<td>Baseline</td>
<td>7.52 ± 3.90</td>
<td>5.73 ± 2.34</td>
<td>5.55 ± 2.10</td>
</tr>
<tr>
<td></td>
<td>1 year</td>
<td>1.78 (0.68, 2.88)</td>
<td>0.18 (-0.34, 0.71)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 year</td>
<td>1.97 (0.96, 2.97)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AST: Aspartate amino transferase, ALT: Alanine amino transferase, TBIL: Total bilirubin, INR: International normalised ratio, UCE: 24 Hour urine copper excretion, NCC: Non ceruloplasmin bound copper, mcg: microgram

**Table 2:** Repeated Measures ANOVA for comparison of Lab parameters between Baseline, 2 year and 3 year post Combination therapy (n=45) and between Baseline, 2 year and 5 years post Combination therapy (n=28)
<table>
<thead>
<tr>
<th>Variables</th>
<th>At Diagnosis/ Follow-up (I)</th>
<th>Follow-up (J)</th>
<th>Mean Difference (I-J) (95% CI)</th>
<th>p Value (Between pairs)</th>
<th>F statistics, p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AST (IU/L)</strong></td>
<td>Baseline 129.38±76.92</td>
<td>3 year 41.78± 14.29</td>
<td>87.60 (58.90, 116.29)</td>
<td><strong>&lt;0.001</strong></td>
<td>57.93 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>2 year 40.86± 11.41</td>
<td>3 year 41.78 ± 14.29</td>
<td>-0.91 (-5.01, 3.18)</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baseline 124.39 ± 82.56</td>
<td>5 year 40.65 ± 11.87</td>
<td>83.74 (42.89, 124.59)</td>
<td><strong>&lt;0.001</strong></td>
<td>28.09 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>2 year 38.11 ± 9.78</td>
<td>5 year 40.65 ± 11.87</td>
<td>-2.54 (-7.83, 2.75)</td>
<td>0.692</td>
<td></td>
</tr>
<tr>
<td><strong>ALT (IU/L)</strong></td>
<td>Baseline 76.58 ± 68.65</td>
<td>3 year 43.80 ± 20.60</td>
<td>32.77 (8.12, 57.41)</td>
<td><strong>0.006</strong></td>
<td>11.59 (0.001)</td>
</tr>
<tr>
<td></td>
<td>2 year 41.85±15.81</td>
<td>3 year 43.80 ± 20.60</td>
<td>-1.95 (-8.86, 4.95)</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baseline 68.57 ± 49.15</td>
<td>5 year 48.41 ± 23.51</td>
<td>20.16 (-7.22, 47.54)</td>
<td><strong>0.013</strong></td>
<td>6.03 (0.014)</td>
</tr>
<tr>
<td></td>
<td>2 year 39.61 ± 14.98</td>
<td>5 year 48.41 ± 23.51</td>
<td>-8.80 (-19.49, 1.89)</td>
<td>0.135</td>
<td></td>
</tr>
<tr>
<td><strong>TBIL (mg/dl)</strong></td>
<td>Baseline 4.41 ± 6.77</td>
<td>3 year 0.85± 0.63</td>
<td>3.55 (1.04, 6.06)</td>
<td><strong>0.003</strong></td>
<td>12.30 (0.001)</td>
</tr>
<tr>
<td></td>
<td>2 year 0.87± 0.56</td>
<td>3 year 0.85 ± 0.63</td>
<td>0.02 (-0.11, 0.15)</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baseline 2.61 ± 2.17</td>
<td>5 year 0.80 ± 0.61</td>
<td>1.81 (0.76, 2.85)</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 year 0.74 ± 0.32</td>
<td>5 year 0.80 ± 0.61</td>
<td>-0.05 (-0.27, 0.16)</td>
<td>19.27 (&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td><strong>Albumin (gm/dl)</strong></td>
<td>Baseline 2.49 ± 0.66</td>
<td>3 year 4.12± 0.43</td>
<td>-1.62 (-1.89, -1.35)</td>
<td><strong>&lt;0.001</strong></td>
<td>180.94 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>2 year 4.10± 0.43</td>
<td>3 year 4.12 ± 0.43</td>
<td>-0.02 (-0.20, 0.15)</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baseline 2.42 ± 0.72</td>
<td>5 year 4.05 ± 0.45</td>
<td>-1.62 (-1.97, -1.27)</td>
<td><strong>&lt;0.001</strong></td>
<td>120.42 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>2 year 4.10 ± 0.33</td>
<td>5 year 4.05 ± 0.45</td>
<td>0.04 (-0.14, 0.24)</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td><strong>INR</strong></td>
<td>Baseline 2.04± 0.68</td>
<td>3 year 1.19± 0.16</td>
<td>0.84 (0.58, 1.10)</td>
<td><strong>&lt;0.001</strong></td>
<td>64.58 (&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>2 year</td>
<td>3 year</td>
<td>5 year</td>
<td>5 year</td>
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</tr>
<tr>
<td></td>
<td>1.20 ± 0.15</td>
<td>1.19 ± 0.16</td>
<td>0.01 (-0.02, 0.05)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.02 ± 0.58</td>
<td>1.18 ± 0.17</td>
<td>0.84 (0.58, 1.09)</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.17 ± 0.12</td>
<td>1.18 ± 0.17</td>
<td>0.00 (-0.06, 0.05)</td>
<td>64.16 (&lt;0.001)</td>
<td></td>
</tr>
</tbody>
</table>

**UCE (mcg/day)**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>3 year</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>629.39 ± 708.77</td>
<td>299.67 ± 171.52</td>
<td>329.72 (82.27, 577.17)</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>322.32 ± 164.64</td>
<td>22.65 (-34.52, 79.83)</td>
<td>2.65 (0.003)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>655.70 ± 774.80</td>
<td>318.78 (-26.56, 664.12)</td>
<td>9.71 (0.003)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>312.13 ± 156.80</td>
<td>-15.79 (-206.08, 174.50)</td>
<td>59.47 (&lt;0.001)</td>
<td></td>
</tr>
</tbody>
</table>

**NCC (mcg/dl)**

<table>
<thead>
<tr>
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<th>Baseline</th>
<th>3 year</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34.10 ± 17.69</td>
<td>1.51 ± 10.56</td>
<td>32.59 (25.63, 39.55)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1.90 ± 12.34</td>
<td>3.99 (5.24, 6.03)</td>
<td>89.03 (&lt;0.001)</td>
<td></td>
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<tr>
<td></td>
<td>33.58 ± 15.45</td>
<td>1.30 ± 4.05</td>
<td>32.27 (25.11, 39.43)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>4.41 ± 13.59</td>
<td>3.10 (-4.16, 10.37)</td>
<td>59.47 (&lt;0.001)</td>
<td></td>
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</tbody>
</table>

**Hemoglobin (gm/dl)**

<table>
<thead>
<tr>
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<th>Baseline</th>
<th>3 year</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>10.19 ± 1.64</td>
<td>12.59 ± 1.17</td>
<td>-2.40 (-3.11, -1.68)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>12.31 ± 1.54</td>
<td>12.59 ± 1.17</td>
<td>-0.28 (-0.70, 0.14)</td>
<td>0.321</td>
</tr>
<tr>
<td></td>
<td>10.30 ± 1.76</td>
<td>12.18 ± 1.30</td>
<td>-1.88 (-2.70, -1.07)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>12.33 ± 1.33</td>
<td>12.18 ± 1.30</td>
<td>0.15 (-0.31, 0.61)</td>
<td>31.64 (&lt;0.001)</td>
</tr>
</tbody>
</table>

**Total Platelet count (Thousands/mm³)**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>3 year</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>132.31 ± 76.34</td>
<td>163.67 ± 82.85</td>
<td>-31.35 (-54.80, -7.91)</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>159.09 ± 92.62</td>
<td>163.67 ± 82.85</td>
<td>-4.57 (-22.21, 13.05)</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>129.89 ± 62.87</td>
<td>163.18 ± 82.00</td>
<td>-33.28 (-67.71, 1.14)</td>
<td>0.061</td>
</tr>
<tr>
<td></td>
<td>156.79 ± 71.02</td>
<td>163.18 ± 82.00</td>
<td>-6.39 (-32.39, 19.61)</td>
<td>4.91 (0.011)</td>
</tr>
</tbody>
</table>

**Total Leukocyte count**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>3 year</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.09 ± 4.29</td>
<td>5.88 ± 1.92</td>
<td>2.20 (0.64, 3.76)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>5.88 ± 1.92</td>
<td>5.88 ± 1.92</td>
<td>2.20 (0.64, 3.76)</td>
<td>11.29 (0.001)</td>
</tr>
<tr>
<td>(Thousands/mm³)</td>
<td>2 year 6.13 ± 2.09</td>
<td>3 year 5.88 ± 1.92</td>
<td>0.24 (-0.37, 0.86)</td>
<td>0.028</td>
</tr>
<tr>
<td>-----------------</td>
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</tr>
<tr>
<td></td>
<td>Baseline 7.13 ± 2.72</td>
<td>5 year 5.63 ± 1.70</td>
<td>1.50 (0.13, 2.87)</td>
<td>0.663</td>
</tr>
<tr>
<td></td>
<td>2 year 6.13 ± 1.99</td>
<td>5 year 5.63 ± 1.70</td>
<td>0.49 (-0.51, 1.50)</td>
<td>5.00 (0.010)</td>
</tr>
</tbody>
</table>

**Abbreviations:** AST: Aspartate amino transferase, ALT: Alanine amino transferase, TBIL: Total bilirubin, INR: International normalised ratio, UCE: 24 Hour urine copper excretion, NCC: Non ceruloplasmin bound copper, mcg: microgram

**Table 3: Comparison of Lab parameters between Patients with Good compliance versus Poor Compliance 2 year post Combination therapy (N=69)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Good Compliance (Mean ± SD) (n=51)</th>
<th>Poor Compliance (Mean ± SD) (n=19)</th>
<th>Mean Difference (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (IU/L)</td>
<td>39.31 ± 11.34</td>
<td>77.52 ± 34.59</td>
<td>-38.21 (-49.09, -27.33)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>40.05 ± 19.62</td>
<td>91.94 ± 52.74</td>
<td>-51.89 (-69.01, -34.76)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>TBIL (mg/dl)</td>
<td>0.84 ± 0.45</td>
<td>1.35 ± 1.77</td>
<td>-0.50 (-1.03, 0.02)</td>
<td>0.062</td>
</tr>
<tr>
<td>Albumin (gm/dl)</td>
<td>4.20 ± 0.39</td>
<td>3.88 ± 0.66</td>
<td>0.31 (0.06, 0.57)</td>
<td>0.015</td>
</tr>
<tr>
<td>INR</td>
<td>1.21 ± 0.15</td>
<td>1.30 ± 0.36</td>
<td>-0.09 (-0.21, 0.03)</td>
<td>0.147</td>
</tr>
<tr>
<td>UCE (mcg/day)</td>
<td>281.26 ± 160.04</td>
<td>560.66 ± 323.62</td>
<td>-279.40 (-395.13, -163.68)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>NCC (mcg/dl)</td>
<td>1.05 ± 10.49</td>
<td>6.33 ± 5.80</td>
<td>-5.27 (-10.36, -0.19)</td>
<td>0.042</td>
</tr>
</tbody>
</table>

**Abbreviations:** AST: Aspartate amino transferase, ALT: Alanine amino transferase, TBIL: Total bilirubin, INR: International normalised ratio, UCE: 24 Hour urine copper excretion, NCC: Non ceruloplasmin bound copper, mcg: microgram
Table 4: Change in 24-hours urinary copper excretion values in those patients who achieved criteria for adequate chelation (excluding 24-hours urinary copper < 500 mcg/day) at 2-, 3- and 5- years follow-up

<table>
<thead>
<tr>
<th>Time</th>
<th>Mean Difference</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>At Diagnosis/Follow-up (I)</td>
<td>Follow-up (J)</td>
<td>(Mean ± SD)</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2 year</td>
<td>589.02 ± 721.37</td>
<td>287.70 ± 162.94</td>
</tr>
<tr>
<td>2 year</td>
<td>3 year</td>
<td>287.70 ± 162.94</td>
<td>305.89 ± 179.02</td>
</tr>
<tr>
<td>3 year</td>
<td>5 year</td>
<td>305.89 ± 179.02</td>
<td>331.18 ± 332.63</td>
</tr>
</tbody>
</table>

Abbreviations: CAC: Criteria for adequate chelation, UCE: 24- hour urine copper excretion

Figures
Figure 1

Trend of various lab parameters (A): Aspartate aminotransferase and alanine aminotransferase, (B) Serum albumin & INR, (C) 24 hour urine copper excretion and (D) Non ceruloplasmin bound copper at the time of diagnosis (Baseline) & various time points on follow up
Figure 2

(A) Kaplan Meier Curves showing proportion of the enrolled cohort achieving the criteria for adequate chelation (CAC) at various time points on follow up (B) Comparison between patients with good compliance versus poor compliance