



ENZOBIOTICS IN ADVANCED CHRONIC KIDNEY DISEASE A DOUBLE BLIND RANDOMIZED CONTROL TRIAL – SUB ANALYSIS OF EETOX STUDY

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ABSTRACT

Dysbiosis in chronic kidney disease (CKD) results in elevated levels of protein-bound uremic toxins (PBUTs), which contribute to insulin resistance, renal function decrease, and cardiovascular disease risk. Enzobiotics, a combination of symbiotics and proteolytic enzymes, are intended to restore gut homeostasis and lower PBUTs in patients with CKD. The objective of this subanalysis of the EETOX trial is to assess the efficacy of Enzobiotics in lowering p-Cresol Sulfate (PCS) and Indoxyl Sulfate (IS) levels while preserving kidney function in patients with advanced CKD. **Methods:** A randomized, placebo-controlled, double-blind study was conducted on 46 patients with stage 4 and 5 CKD. Patients were divided into Enzobiotics and placebo groups, and PBUT levels, serum creatinine, urea, potassium, and estimated glomerular filtration rate (eGFR) were recorded at baseline and 90 days. **Results:** The Enzobiotics group showed an 18% decrease in IS

levels, while the placebo group exhibited a 52% increase ($p < 0.01$). PCS levels declined by 35% in the Enzobiotics group, compared to an increase in the placebo group ($p < 0.01$). Enzobiotics therapy also slowed the rate of urea increase (1% vs. 8%), stabilized creatinine levels, reduced potassium by 2%, and slowed eGFR decline (8% vs. 13%, $p < 0.01$).

Conclusion: Enzobiotics effectively reduces PBUT levels and helps preserve renal function in advanced CKD patients. By mitigating inflammation and stabilizing kidney parameters, Enzobiotics may serve as a promising intervention to delay CKD progression and postpone the need for dialysis.

KEYWORDS: Enzobiotics, p-Cresol Sulfate, Indoxyl Sulfate, Stage 5 CKD, Creatinine, Urea.

INTRODUCTION

Dysbiosis, characterized by imbalances in gut microbiota and disruption of the gut barrier, is marked by increased concentrations of pathogenic Pro-inflammatory bacteria. This imbalance contributes to loss of endothelial integrity, heightened intestinal permeability and increase in levels of protein-bound uremic toxins (PBUTs) contributing to insulin resistance, progressive decline in kidney function, and elevated cardiovascular disease (CVD) risk. Serum PBUTs like IS & PCS levels rise with the worsening severity of chronic kidney disease (CKD).

Enzobiotics, a combinational therapy of symbiotics and proteolytic enzymes, aims to induce favorable changes in the uremic gut environment, promoting near-normal protein assimilation and restoring a Eubiotic gut state in CKD patients.

AIM

This study is a sub analysis of the EETOX study, This sub analysis was considered as almost all patients were in advanced CKD and had comparable Ethnicity, Culture, Food habits, Geographical position and same baseline treatment protocols. 46 patients were part of this two center trial. The trial focused on evaluating the efficacy of Enzobiotics in reducing the generation of protein-bound uremic toxins, p-Cresol Sulfate (PCS) and Indoxyl Sulfate (IS) among patients with advanced renal failure. The study also aims to assess role of Enzobiotics on serum creatinine, phosphorus, potassium levels, and its effectiveness in preservation of renal function, as measured by the estimated glomerular filtration rate (eGFR) using the MDRD equation, among patients with stage 4 and 5 chronic kidney disease (CKD)

BACKGROUND

Chronic kidney disease (CKD) is increasingly prevalent and significantly impacts quality of life, leading to high morbidity and early mortality^[1,2] Kidneys play a crucial role in protein metabolism, primarily by handling amino acids essential for tissue repair and maintenance.^[3]

Dietary protein intake has a profound negative effect on renal function.^[5] High-protein diets, commonly used for weight management and diabetes control, can adversely affect the gut environment by inducing dysbiosis, disrupting metabolic pathways and compromising mucosal homeostasis in the large intestine leading to intraglomerular hypertension, proteinuria, and renal hyperfiltration.^[3,6,7]

Highly bioavailable animal proteins, tend to exacerbate gut dysbiosis and inflammation more than plant proteins.^[8,9,10,11] Animal protein increases the production of nitrogenous end-products, worsening uremia and causing constipation.

In CKD, a reduced protein intake of 0.6-0.8 g/kg/day is recommended to mitigate these adverse effects.^[1,5] Muscle protein synthesis is optimized with an intake of 20-25 grams of high-quality protein, excess being oxidized for energy or converted into urea and other organic acids.^[4]

Dysbiosis impairs protein assimilation, leading to the accumulation of undigested proteins in the distal intestine, increasing protein fermentation producing toxic metabolites like ammonia, phenols, amines, indoles, and thiols.^[12,15] Protein-bound **uremic toxins** (PBUTs) like p-Cresol Sulfate (PCS) and Indoxyl Sulfate (IS) are generated as byproduct of protein fermentation.

IS, derived from tryptophan, is associated with atherogenesis, vascular inflammation, and oxidative stress, while PCS, derived from tyrosine, primarily elicits pro-inflammatory responses.^[19,20] Inflammation and fibrosis are major factor contributing to progression of CKD. CKD is also compounded by gut edema, systemic congestion, slow transit, metabolic acidosis, decreased dietary fibre intake, insulin resistance.^[17,21,22] Treatment strategies for CKD should focus on slowing disease progression rather than dialysis and kidney transplantation.

Restoring gut microbiota through Synbiotics and Enzobiotics presents a promising therapeutic approach.^[24,25]

The objective of this study is to evaluate

- Enzobiotics (proteolytic enzymes + symbiotics) role in reducing protein-bound uremic toxins.

- Enzobiotics usage for retarding the progression of CKD, assessed by the Creatinine, Urea & eGFR (MDRD) among patients with Stage 4 and 5 CKD.

MATERIALS AND METHOD

46 CKD stage 4 & 5 patients were randomized to receive Enzobiotics or placebo for a period of 90 days. Patients were recruited from, KG PG Institute multipurpose hospital, Coimbatore and Dr Sanjay's Center for Kidney and Diabetes, Bangalore from south India. In this double blinded, randomized control, Prospective trial, both patient and physicians were blinded to treatment received by the subjects. Both the Enzobiotics and Placebo medications had identical package, prints, color, form, size, strips, and boxes to maintain the blinded status. Randomization was done using computer-generated random numbers.

Study Participants and Intervention

Patients were randomised into Group A-Enzobiotics treatment, and Group B- the Placebo group. Under the guidelines of CTIA India, The Hague declaration, and the Hospital Ethical committee, patients were enrolled following consent for a follow-up period of 90 days.

Totally 46 patients were recruited from the two centers, 29 were allocated to Enzobiotics group and 17 to the placebo group. Enzobiotics capsules contained 250 mg each of Lactobacillus, Bifidobacterium, and Streptococcus genera, with 100 mg of prebiotic, and 150 mg of the proteolytic enzyme with an activity of 3,55,000 IU. Placebo was Maltodextrin capsule in the dosage of 500mg. Trial drug & Placebo were given three times a day, five minutes before each main meal as per the allocation. Physical parameters and levels of P Cresol Sulphate, Indoxyl Sulphate, Urea, Creatinine, Electrolytes and eGFR estimated by MDRD were recorded at baseline and again at the end of 90 days of therapy for each of the patient in both the groups. The parameters recorded at initial assessment (Day 0) and of final assessment (Day 90) were compared and analyzed.

Ethical Consideration

The trial is registered with CTRI/2019/01/017070 under protocol number Nizy-Byt-Mb-18. Trial is authorized by the ethics committees of the Institutes after reviewing study-related documents. Written consent was obtained before enrolling subjects for the study, in compliance with the Helsinki Declaration.

Inclusion and Exclusion criteria

Subjects between the age group of 18 and 70, having stable Stage 4 and 5 CKD, willing to participate in study with a written informed consent were included in the study. Patients with HBsAG, anti HCV, HIV positive status, Ongoing infection or history of recent infections were excluded from the study. Nursing women, women wanting pregnancy, patients with autoimmune disease like SLE, vasculitis, patients with hepatic impairment (SGOT or SGPT levels >3 times the upper limit) and those with a diabetic foot infection, unstable cardiovascular history, patients with severe systemic illness, and those who were already on synbiotics or those allergic to synbiotics or proteolytic enzymes were excluded from the trial. All adverse events (AE) and serious adverse events (SAE) were recorded and documented.

Study Design

Each patient received a unique identifying number, and the medications were recorded in a log. Each patient had total of four visits, including the initial screening, day 0 followed by visits on day 45 and day 90. All participants had the right to withdraw from the study at any time. Subjects who could not adhere to the protocol and analysis criteria were excluded. Measured physical parameters included: weight, Height, BMI, Blood Pressure, Respiratory rate, Heart rate and Pulse rate. In compliance with the Declaration of Helsinki, patients were asked to return any unused medication. Drug records were maintained meticulously. The investigation product (IP) was administered on each visit.

Biochemical parameters - CBC, Urea, Creatinine, Electrolytes, Bilirubin, SGOT, SGPT, PUBTs (PCS&IS), Lipid Profile were also measured and recorded; eGFR was calculated using the MDRD and Cockcroft Gault formulas.

To prevent bias, diet of all the participants was maintained unchanged.

Clinical adverse event, laboratory anomaly, concomitant illness, other medical condition or circumstance resulting in the termination of the study subject from further participation in the trial were recorded. The patients were eliminated from trial & analysis if lost to follow-up, loss of compliance, disregard to the protocol, or if a concurrent disease impacted subjects health.

Sample Analysis

Serum isolated from 10 ml of blood sample was taken and used for quantifications of Indoxyl sulfate and p Cresyl sulfate, analysed at Notrox Research.

For p-cresyl sulfate, 200 mcl of serum was combined with 1,000 mcl of Acetonitrile that contained Itraconazole.

For Indoxyl Sulfate, 200 mcl of serum was combined with 200 mcl of Acetonitrile that contained Obeticholic acid. Protein was separated by Centrifugation at 4,000 rpm for 20 minutes at 4 °C and then the supernatant was collected for LC-MS/MS bioanalysis.

In blank charcoal-stripped serum, the calibration curve had 8 points and ranged from 105.790 to 95283.170 ng/ml. The central laboratory at SRL, Mumbai processed and analyzed all the other parameters.

The STARD guidelines were followed for the diagnostic accuracy of PCS and IS by a NABL-accredited Laboratory, Notrox Research Private Limited Bengaluru.

To eliminate bias, biochemical measurements were analysed in a central laboratory with reference limits defined under laboratory standards.

Statistical Analysis

The two-sample T-Test was used to assess the ratio of increase in PCS and IS between Day 90 to Day 0 and then compared between the Interventional product Enzobiotics and the Placebo. After confirming normality, it was utilized to determine the significance of differences between the two groups in terms of the Day 90/Day 0 ratio. The initial observations (Day 0) between Placebo and Enzobiotics were analyzed for normality and homogeneity of variance (F-ratio). Utilizing capability analysis, the results were compared to the normal range after treatment to identify any potential concerns. Using multiple regression analysis, the uremic toxins p-Cresyl Sulfate and Indoxyl Sulfate were predicted. The level of toxins and other renal parameters were compared between the initial measurement (Day 0) and day 90.

IS level of 20,000 ng/ml was identified as the thresholds for the risk of CKD progression. The people who did not meet the conditions for analysis were removed to protect the accuracy and validity of observations.

RESULTS

This study focused on the role of Enzobiotics therapy among patients with stage 4 and 5 CKD.

Indoxyl Sulphate

Both the treatment group (Enzobiotics) and the placebo group were found to be largely consistent at the beginning of the study.

The threshold limit for uremic toxin Indoxyl sulfate was set at 20000 ng/ml.

The significant regression coefficient showed that the Enzobiotics group experienced a drop of 18% in level of IS from day 0 to Day 90, contrary to an increase of 52% in the Placebo group. The association was found to be statistically significant ($p < 0.01$), demonstrating how the initial values at day 0 were regulated in the Enzobiotics group and increased in the placebo group. The significance of reduction is much more pronounced in the Enzobiotics group when the initial toxin at day 0 exceeds above threshold level above 20000ng/mL.

Employing the Statistical Linear Regression line, it was found that among the Enzobiotic group, the rate of increase of IS was slow and retarded.

The slopes for rate of change in IS levels for both the groups is shown in Fig 1

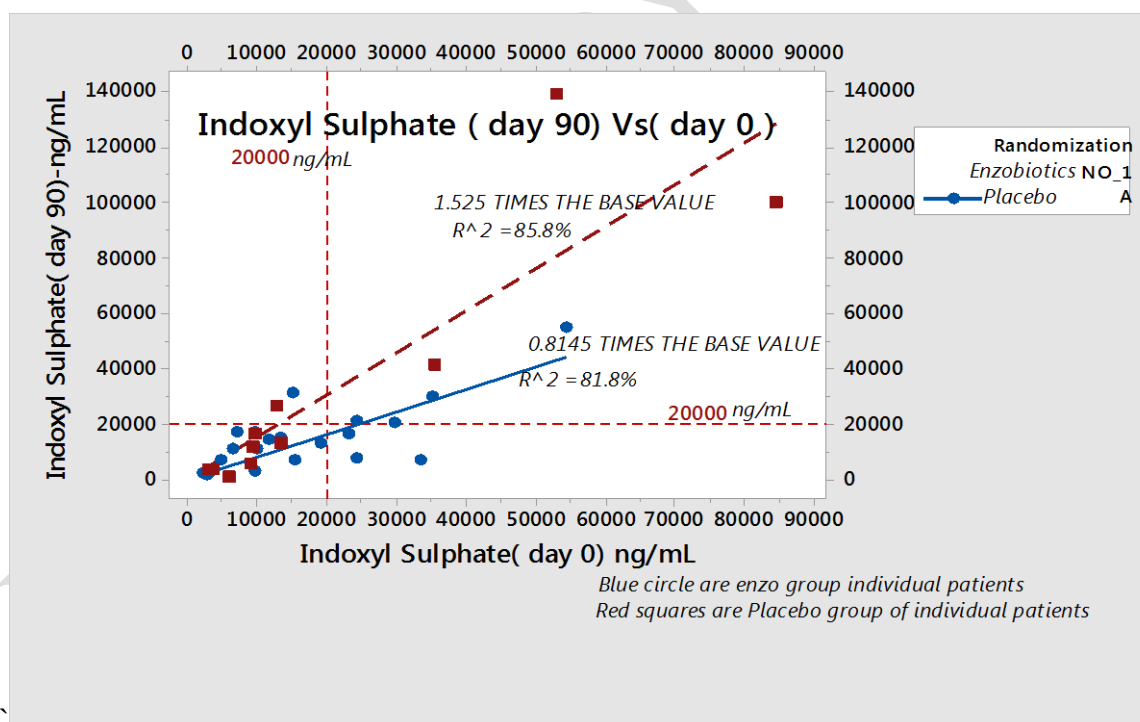


Fig 1: Impact of Indoxyl sulfate on RBC.

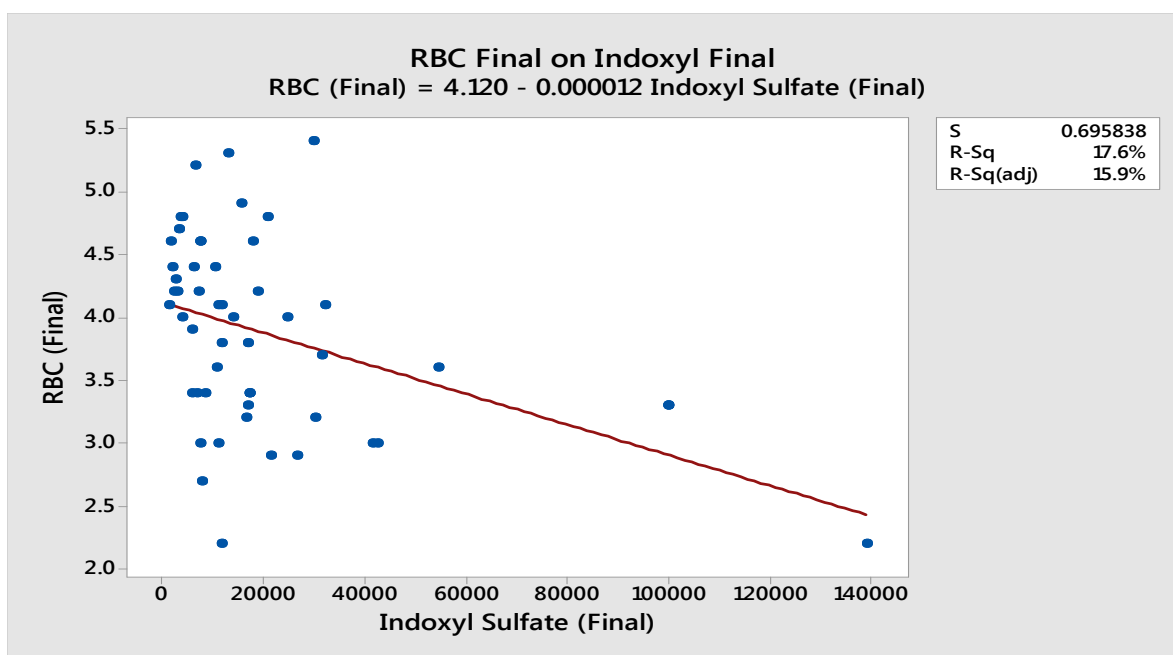


Fig 2

RBC count showed negative linear correlation with IS ($P=0.022$), similar negative linear relationship has been recorded in earlier studies.^[27,28] However with Enzobiotic treatment the slope changes in IS levels was much steeper. These results would translate to better erythropoiesis with Enzobiotics in CKD, however needs validation in future studies.

p-Cresol Sulfate

The cutoff value for p Cresol sulfate was fixed at 20 mcg/ml. The Enzobiotics group experienced a discernible decline when initial p-Cresol was greater than 35 mcg/ml. The outcome of the regression analysis showed that the Enzobiotics group experienced a 35% drop in the levels of PCS.

There was significant association between day 0 and day 90 levels of PCS in both the groups ($p < 0.01$). The significance of the decline in the Enzobiotics group is well appreciated when the toxin at day 0 exceeds the threshold level over 20mcg/ml.

The initial values at day 0 were regulated in the Enzobiotics group and increased in the placebo group.

The Statistical linear regression line showed rate of rise of PCS in Enzobiotics group was much slower and delayed compared to Placebo group.

The slopes for rate of change in PCS levels for both the groups is shown in Fig 3

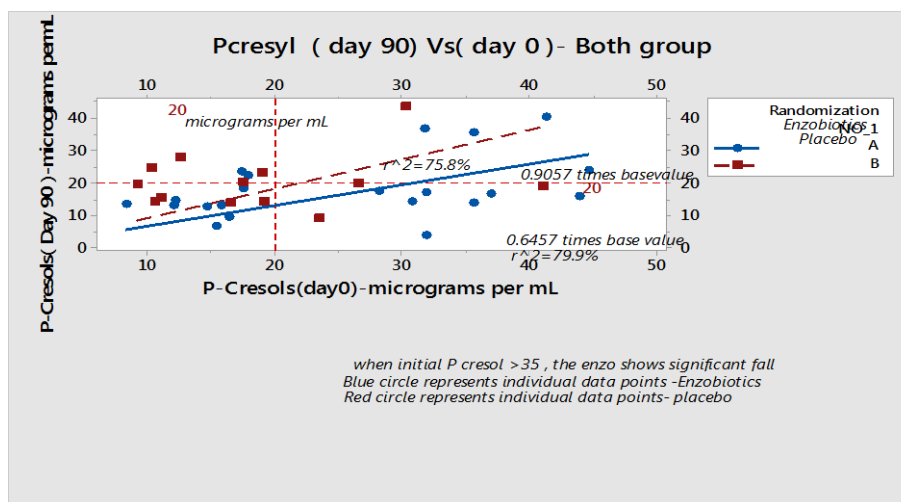


Fig 3.

Estimated GFR (eGFR)

Enzobiotics was found to retard progression of CKD by restricting the decline in GFR, calculated using MDRD to 8% at Day 90 compared to 13 % in the placebo group, indicating the value of Enzobiotics in management of even advanced CKD patients.

The R square value for the two groups was found to be significant ($p < 0.01$) and proved that the eGFR value was considerably higher in the Enzobiotics group compared to the control even in advanced CKD.

Correlation of Urea between the two groups is shown in Fig 4

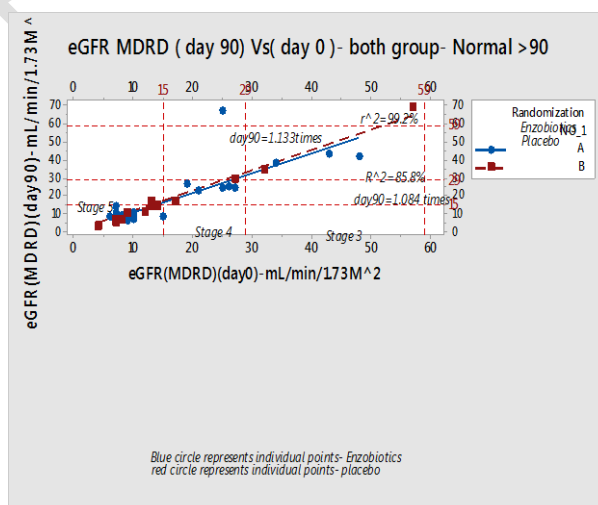


Fig 4

Serum Urea

Urea levels at the initial stage in both groups was consistent, and the rate of increase was slower in Enzobiotics group compared to the placebo group. Only a 1% increase from day 0 was shown by the regression coefficient in the Enzobiotics group, compared to 8% increase in the Placebo group indicating a lower urea level in the Enzobiotics group. In both groups, there was a statistically significant correlation between days 0 and Day 90 ($p < 0.01$). The relevance of the decline in the Enzobiotics group was far greater than the threshold value of 20 mg/dl.

Correlation of Urea between the two groups is shown in Fig 5

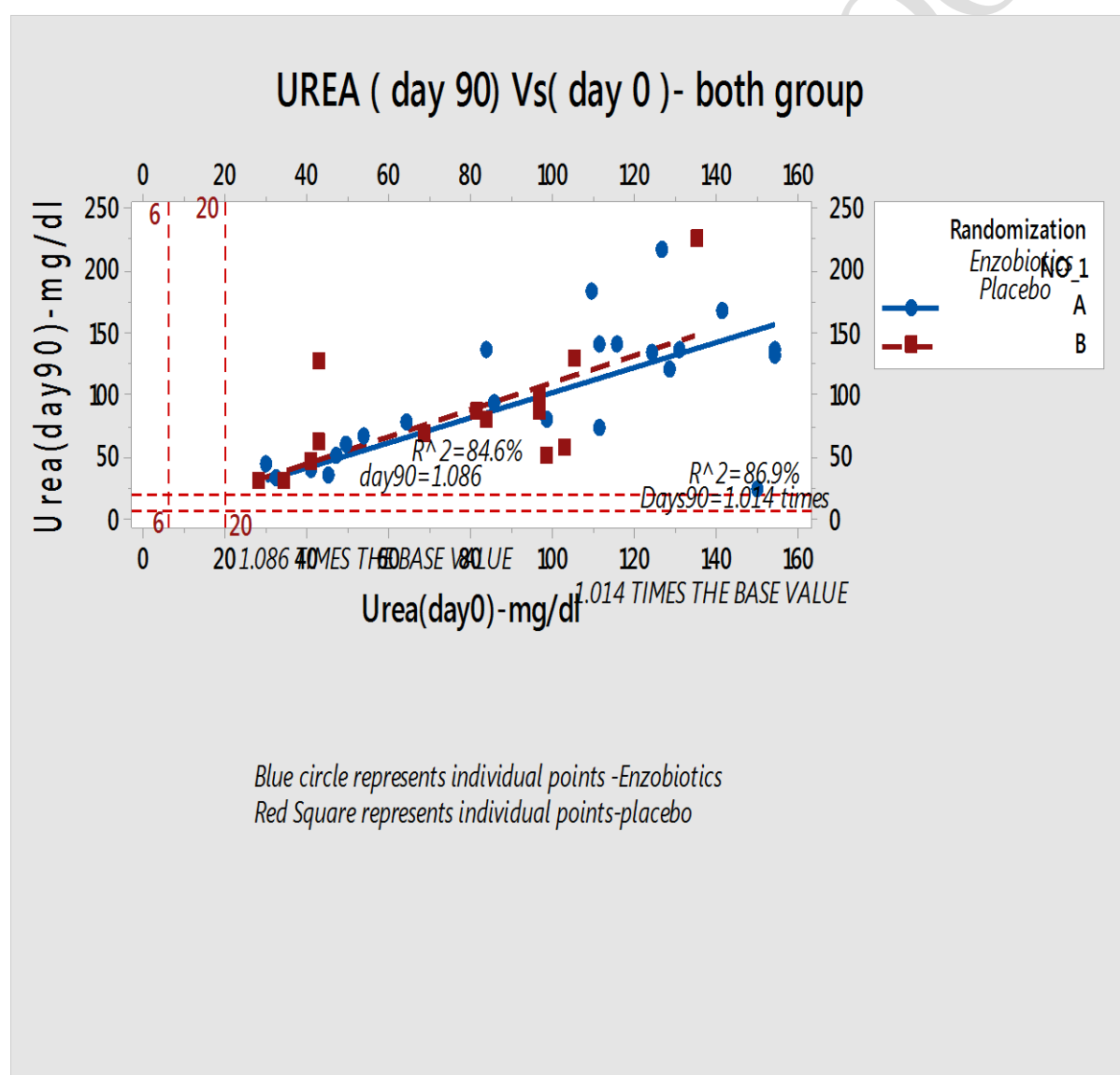


Fig 5

Serum Potassium

The potassium threshold values ranged from 3.5 to 5.1/L. The rate of increase in Potassium level with the Enzobiotics group was lesser & slower than the placebo group. There was a strong correlation between day 0 and day 90 in both groups ($p < 0.01$). Although the early observations in both groups fall within the normal range of 3.5 to 5.1/L, the observations above 5.1/L showed a 2% decrease in Enzobiotics and a 1% increase in placebo. Over the course of 90 days, there was more consistency in the Enzobiotics group than in the placebo group with respect to potassium levels.

The change in potassium levels between the groups is shown in Fig 6

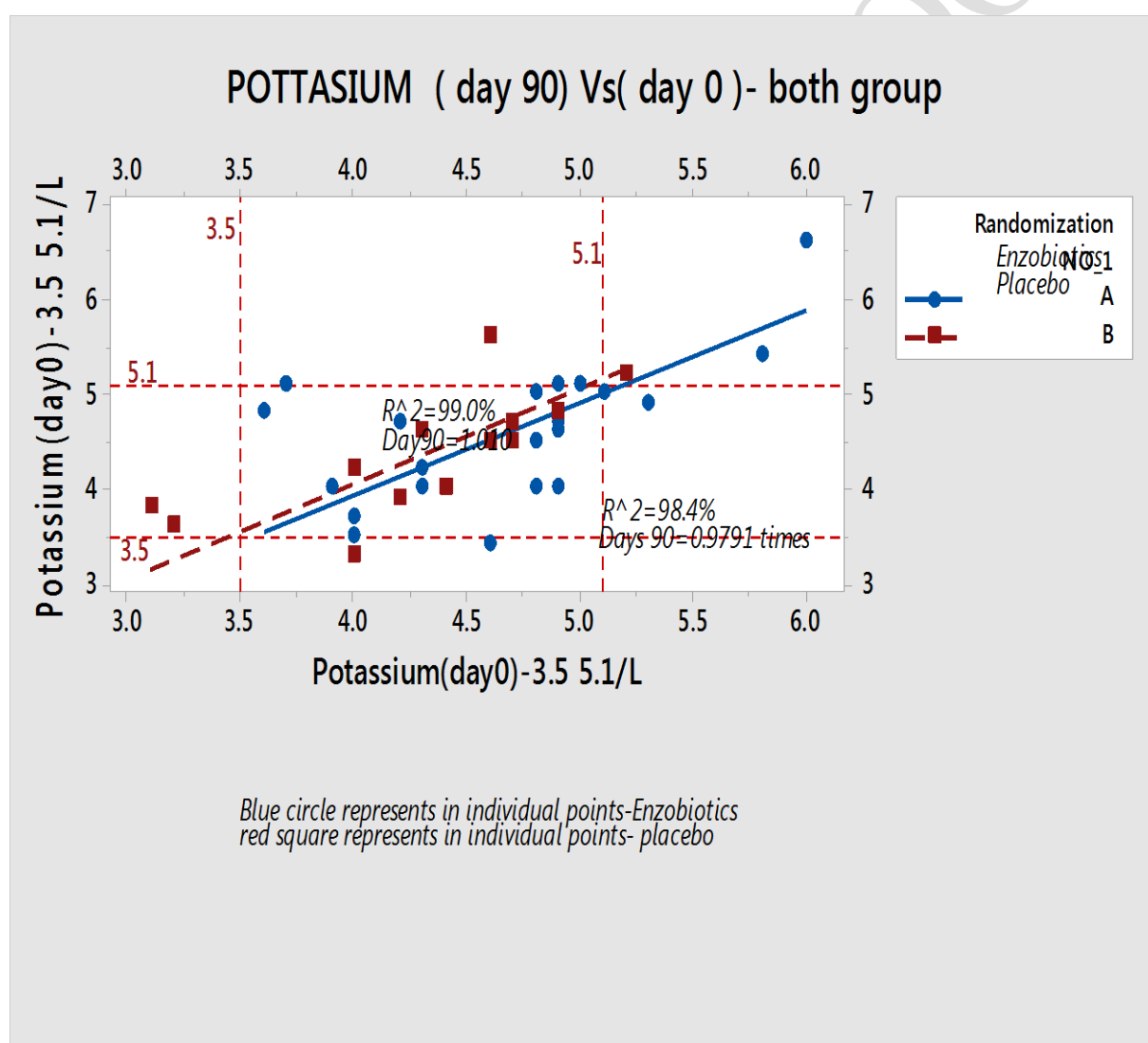


Fig 6

Serum Creatinine

Serum creatinine is the most widely used indicator of renal function. The range of Creatinine threshold limits was 0.9–1.3 mg/dL. Day 0 revealed identical early stages in both groups, and the progression in Enzobiotics group was slower than the placebo group. When Creatinine at day 0 was greater than 6, the Enzobiotics group showed a significant decrease. The level in the Enzobiotics group remained constant, even though it was 8% higher on day 0 than the placebo group, based on the regression coefficient. The R square value was higher in both groups, indicating that the difference between days 0 and 90 was significant for both groups ($p < 0.01$) and that the decline in creatinine level was notable in the Enzobiotics group.

The slope of creatinine for both the groups is shown in Fig 7.

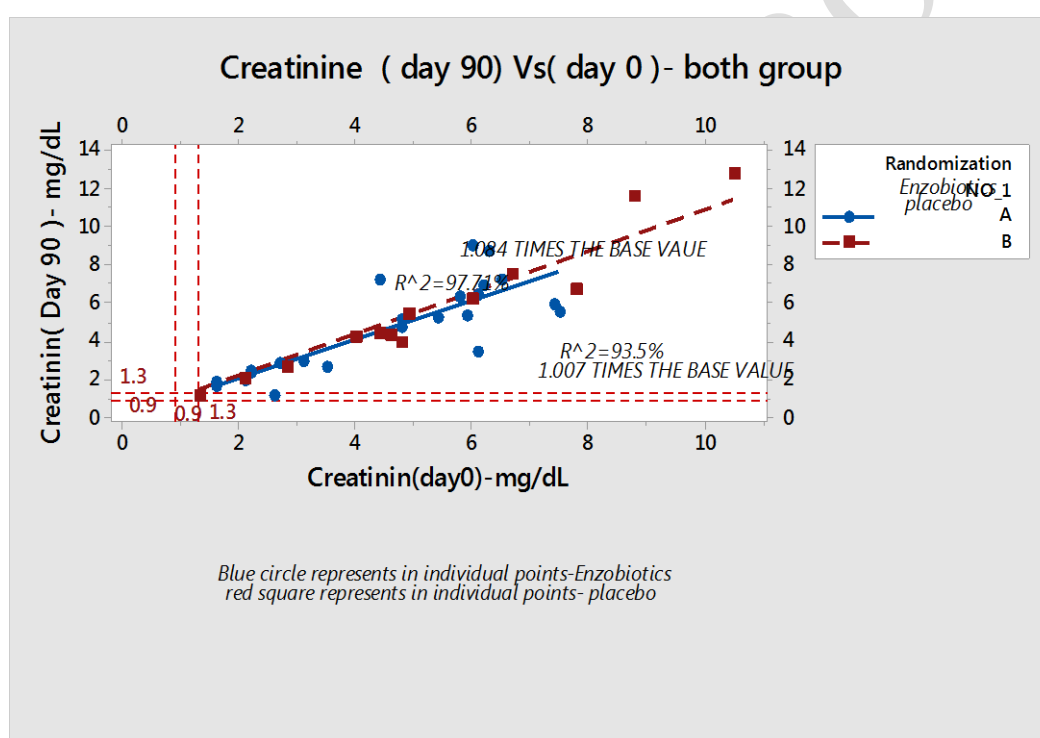


Fig 7.

All crucial parameters pertaining to CKD were kept under control without significant increase in the Enzobiotics group compared to the placebo group.

One of the significant findings of this two-center analysis, not been recorded earlier in any studies was ability of Enzobiotics in delaying progression of CKD even in later stages.

Note that this analysis has been found to be significant with just three months of therapy opening a possibility of better response if therapy is continued for a longer duration.

DISCUSSION

India is a country with varied geography, diverse culture, Ethnicity and Dietary patterns. This sub analysis included patients from the two south Indian centers as the Ethnicity, Culture, Food habits, Geographical position and the baseline treatment protocols of the two centers were very similar and comparable, additionally both these centers contributed to maximum number of patients in stage 4 & 5 CKD.

Enhanced generation of uremic toxins from the dysbiotic microbiome accompanied by reduced renal clearance, causes accumulation of toxic metabolites in blood and other metabolic compartments and consequently progression of CKD to end-stage renal disease (ESRD). Protein intake is associated with increased urinary albumin excretion, elevated creatinine with interstitial fibrosis & tubular atrophy.^[1]

Asian Kidney Disease Study (AKDS) and the Singapore Kidney Function Study (SKFS), assessment on predialysis patients indicate that the protein intake of most CKD patients exceeds the recommended guidelines.^[26] Evidence clearly implicates high protein consumption for renal function decline and progression of CKD.

Low protein diet supplemented with synbiotic therapy can provide a synergistic effect on reducing proinflammatory & profibrotic toxins in CKD patients.^[27,28,29]

In animal studies High protein diet is documented to have 60–70% rise in renal and glomerular volumes, with 55% increase in fibrosis, and 30% increase in glomerulosclerosis in pigs.^[30] Similarly mice on protein rich diet providing 52% of daily energy needs for a period of 24 weeks, causes increase in albuminuria and blood urea nitrogen, implicating protein in diet for renal dysfunction through increased gastrointestinal permeability, and systemic inflammation with urea cycle pathway.^[1]

ARIC study (11) and a population based study from Korea, established role of high protein intake in high incident risk for CKD and rapid decline in eGFR.^[31] In both animal and human models studies have shown high protein diet to be detrimental in developing incident CKD and for progression of CKD.

Managing optimal protein metabolism would be one of the steps in management of CKD & CKD progression.

Proteinase, an enzyme to break complex proteins into 1 or 2 chain amino acids for complete absorption of protein from gut is a new concept for amelioration or better outcome of CKD.

Proteolytic enzymes have shown better protein assimilation and reduced inflammation with improvement in many key nutritional indices among CKD patients.^[35,36]

Symbiotics have shown to improve inflammatory status and improve renal function in adenine-induced CKD rat model.^[33] Sanjay et al have recorded significant reduction in inflammatory markers and improved QOL among patients with stage 5D CKD.^[37] Prebiotic, Probiotic, and Symbiotic induces restoration of gut microbiome of CKD patients^[14] and contributes to better outcomes in both non dialysis and dialysis CKD patients.^[40]

The synergy study reported role of probiotics in significant reduction of serum PCS and alteration of the stool microbiome with enrichment of Bifidobacterium and depletion of Ruminococcaceae.^[42]

Dysbiosis & Incomplete protein metabolism are the two most important factors contributing to progression and maintenance of CKD.

Enzobiotics a combination of Protease enzyme & Symbiotics is a probable solution which tackles both these issues by better Protein assimilation and inducing gut Eubiosis, reducing generation of PUBTs and bringing CKD gut closer to normal intestinal milieu. Treatment with Enzobiotics improves integrity of gut epithelium and reduce inflammatory and profibrotic cytokines with improved outcome in CKD.

Enzobiotics trial among gentamycin induced CKD rats had shown a significant decrease in urine protein excretion and serum creatinine levels. With histological evidence of renoprotection and regeneration.^[32]

The complete mechanism of action of Enzobiotic is discussed in EETOX study published in Nutrients 2022.^[34] P Cresol sulfate & Indoxyl sulfate the predictive markers for decline in renal function was found to decrease with use of Enzobiotics among patients with stage 3 to 5 CKD in EETOX study.^[34]

This subgroup analysis among advanced CKD patients showed reduction in IS levels by 18% much more compared to 6.7% recorded in EETOX study.

The reduction in PCS was much more in this study 35% compared to EETOX 31%.

For the first time Our study recorded increase in GFR by 8% and stabilization of serum creatinine levels among patients with severe renal failure. Creatinine was recorded to stabilize or remain same during three months of Enzobiotics treatment, despite being 8% higher than placebo group at day 0. Enzobiotics were found to have beneficial effect even on potassium levels. Regression coefficient of all parameters were recorded to be statistically significant ($p < 0.01$).

This analysis has again showed the beneficial effect of two step correction of CKD milieu using Enzobiotic, the new novel treatment option for CKD. This analysis has also shown that the response and beneficial effects of Enzobiotic is much more pronounced in advanced CKD.

Enzobiotic may help to retard progression of CKD and would probably help delay initiation of dialysis among patients with ESRD.

Progression of CKD may be modified by dietary approaches aimed at altering the gut microbiota and the enzymatic activities of its proteome (Diet–microbiota interaction and kidney disease progression).^[43]

Enzobiotics may hold key to better outcome in CKD as it tackles both the issues of protein metabolism and dysbiosis, the two major triggers for inflammation and oxidative stress in CKD.

Enzobiotics may be a new therapeutic option to delay progression of CKD, improve QOL and improve anaemia management in CKD.

Enzobiotic seems to have much more pronounced beneficial effect in advanced CKD. Validation of these results would be needed with a larger study over a longer period.

CONCLUSION

Enzobiotics is a good Conservative treatment option for patients with severe CKD and among patients not willing for Dialysis.

Enzobiotics delays progression of CKD and can be used as a option for delaying Dialysis.

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