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The effect of squalene on proteinuria in patients with type 2 diabetes mellitus

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ABSTRACT

Background: Squalene, in recent years, has become a topic of interest to scientists due to its potential health benefits and anti-inflammatory effects. Squalene is a hydrocarbon belonging to the triterpene class; it is a 30-carbon isoprenoid compound. In previous studies, amaranth oil, containing high amounts of squalene, was shown to function as an effective treatment option for reducing proteinuria, one of the key markers for renal disease.

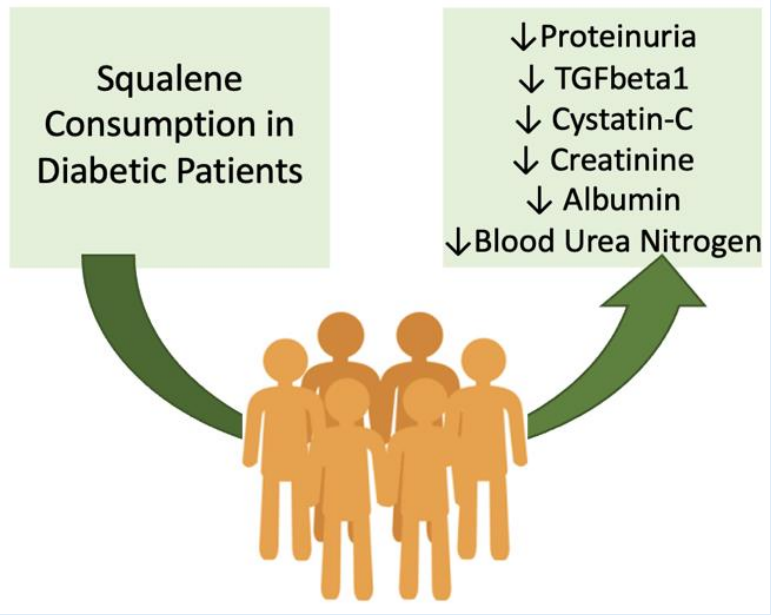
Objectives: Our main goal was to understand the effect of squalene as a biotic agent for reducing proteinuria. In order to identify squalene as the decreasing agent of proteinuria in amaranth oil, we conducted an experimental study on diabetic patients. Our research focused on patients with type 2 diabetes mellitus with separate dosages of squalene serum consumption. Over the course of 84 days, we tracked changes in proteinuria levels based on the dosage of squalene consumed. In addition to this, testing was also conducted for 84 days on various related parameters. These parameters include blood urea nitrogen (BUN), cystatin C, albumin, systolic and diastolic blood pressure, and transforming growth factor b1 (TGFB1). By assessing changes throughout the study, we hoped to analyze the relationship between proteinuria and these associated parameters.

Methods: Five groups of 30 people, totaling 150 volunteers, were recruited into the study. In group 1, 30 healthy people served as the healthy group. In group 2, 30 T2DM patients did not consume any squalene, thereby serving as the diabetic control group. Group 3 consisted of 30 T2DM patients who consumed 200 mg of squalene (extracted from shark liver) daily. Group 4 consisted of 30 T2DM patients who consumed 400 mg of squalene daily. Lastly, group 5 consisted of 30 T2DM patients who consumed 600 mg of squalene daily. In groups 3, 4, and 5, the patients were prescribed to consume squalene for a total of 84 consecutive days. Among the patients with type 2 diabetes referred to Vali-Asr medical laboratory (Tehran, Iran), 120 were selected. Also, 30 people participated in the study as healthy individuals. Regarding World Health Organization, inclusion criteria included the following: fasting plasma glucose amounts ≥ 126 mg/dL, glycated hemoglobin (HbA1c) ≥ 6.5%, and not taking corticosteroids. Informed consent was obtained from all volunteers.

Results: Throughout these 84 days, proteinuria levels decreased in these patients with high statistical significance. A positive correlation was also observed with dosage amount, as there had been a higher level of decrease in proteinuria amount with a higher dosage of squalene serum administered for consumption. Statistical significance was found in proteinuria as well as the associated parameters tested (BUN, Albumin (AL), creatinine (CR), cystatin C, and TGFbeta1.

Conclusion: All of the associated health indicative parameters we tested alongside proteinuria count also showed a trend of overall reduction throughout the duration of 84 days. These values vary based on the dosage of squalene consumed as well as the time elapsed from the initial prescription. Based on these results, it may be assumed that squalene functions effectively to reduce all of these parameters as well as overall proteinuria level.

Keywords: Squalene, proteinuria, renal disease, diabetes, functional food, amaranth oil



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INTRODUCTION

Squalene is a hydrocarbon made up of a 30-carbon ring and belongs to the triterpene class [1]. It is a 30-carbon isoprenoid compound. Squalene is commonly found naturally in shark liver oil, olive oil, and some vegetable oils [1]. Amaranth oil, which contains high amounts of squalene, was found in previous studies to reduce proteinuria levels [2]. Proteinuria refers to the presence of protein found in the urine [3]. This acts as a health indicator that there is damage to the kidney [4]. The protein commonly found in proteinuria is particularly albumin, which is found in urinary excretion [11]. Normal protein levels found in urinary analysis are values less than 150 milligrams in the last 24 hours [3]. When the value is higher than or equal to 150 milligrams in the last 24 hours, this indicates the presence of proteinuria [3]. A value received higher than 3500 milligrams in the last 24 hours indicates nephrotic proteinuria [3].

Current treatments for proteinuria include medication intake, change in diet, and change in exercise. For individuals with high blood pressure or diabetes, the focus is on anti-hypertensive treatments. One specific form includes treatment using both an ACE inhibitor along with an ARB [5]. ACE inhibitors function to disrupt the renin-angiotensin-aldosterone system, thereby acting as an anti-hypertensive option [6]. This way, the ACE inhibitor assists in the minimization of protein in urinary excretion [12]. Squalene is a growing topic of interest due to recent findings of a multitude of health benefits.

Intriguingly, squalene may possess anti-cancer properties. According to a previous study, it was shown that squalene functioned successfully in lieu of mineral oil for treatment of tumors with *Bacillus Calmette-Guerin* cell walls in mice [7]. This anti-cancer property of squalene calls for future investigation of drug development. Another health benefit that squalene possesses is cardioprotection. In mice administered with squalene, squalene exhibited antioxidant effects and served as a defense for the heart [8]. It was also found that squalene functioned effectively in protecting DNA and reducing reactive oxidative species [13].

In order to identify squalene as the proteinuria reducing agent in amaranth oil, we created an experiment analyzing these values. For our study, we focused on studying proteinuria levels with squalene administration in diabetic patients. The goal of our study was to better understand the effects of serum squalene on proteinuria count in patients with Type 2 diabetes. By understanding the relationship between squalene and proteinuria, we can identify a new potential treatment option for those suffering from renal disease. In addition to measuring proteinuria count, we also collected data regarding changes in proteinuria related to variables such as blood urea nitrogen (BUN), creatinine, cystatin C, systolic and diastolic blood pressure, albumin, and TGF-B. Creatinine-proteinuria and albumin-proteinuria ratios are both used for proteinuria level analyses [15]. It has been observed that the total protein:creatinine ratio is more effective for analysis than the albumin-creatinine ratio [15]. Previous research has indicated that cystatin C levels may be impacted by proteinuria. It was found that cystatin C in urinary excretion changed with the presence of proteinuria [9]. In terms of relation to BUN, it was found in a previous study that with the treatment of vitamin E, Streptozotocin-induced diabetic rats had shown a reduction in proteinuria and BUN [10].

Through our current study, we can observe whether or not proteinuria and BUN show a similar connected trend. Previous research on the relationship between albuminuria and proteinuria provides increased interrelatedness. A study showed that in their sampling, they found that of the individuals they tested with proteinuria, 91% also had albuminuria [14]. The converse relationship, however, lacked correlation, as only 32% of those with albuminuria tested also had proteinuria. Our aim in comparing the variables with proteinuria levels was to observe any trends in data over the course of this study with squalene consumption. By doing so, we can analyze

these relations to generate future treatment options for proteinuria.

MATERIALS AND METHODS:

Squalene (S3626) was purchased from Sigma Company (USA). The assay kits of proteinuria, albuminuria, and cystatin C were procured from Abcam Company (USA). The serum concentrations of BUN and creatinine were determined by the assay kits purchased from ARBOR ASSAYS Company (USA). The Human TGF-b1 assay kit was procured from Boster Biological Technology company (USA).

In this randomized study, five groups of 30 people, totaling 150 volunteers, were recruited to the study. In group 1, 30 healthy people served as the healthy group. In group 2, 30 T2DM patients did not consume any squalene, thereby serving as the diabetic control group. Group 3 consisted of 30 T2DM patients who consumed 200 mg of squalene daily. It should be noted that the treated groups received squalene as an oral capsule (liquid filled oral). Group 4 consisted of 30 T2DM patients who consumed 400 mg of squalene daily. Lastly, group 5 consisted of 30 T2DM patients who consumed 600 mg of squalene daily. In groups 3, 4, and 5, the patients were prescribed to consume squalene for a total of 84 consecutive days. Among the patients with type 2 diabetes referred to Vali-Asr medical laboratory (Tehran, Iran), 120 were selected. Also, 30 people participated in the study as healthy individuals. Regarding World Health Organization, inclusion criteria included the following: fasting plasma glucose amounts ≥ 126 mg/dL, glycated hemoglobin (HbA1c) ≥ 6.5%, and not taking corticosteroids. Thus, people with T2DM, without any other disease, were included in the study. Exclusion criteria in this study were people without T2DM and people who had the metabolic diseases and had a history of surgery, as well as smokers. Informed consent was obtained from all volunteers.

General features and sampling: Blood samples were obtained from volunteers under sterile conditions. Sampling was performed in five time periods on days 1, 14, 28, 56, and 84. In each period, anthropometric items including age, sex, weight, height, body mass index systolic blood pressure, and diastolic blood pressure of all study participants were recorded. After 12 hours of nighttime fasting, blood samples were taken from all groups. These samples were centrifuged (250 g for 10 min) to prepare serum samples. Separated serum samples were used to measure the biochemical parameters.

Biochemical measurement: Biochemical parameters of all groups were analyzed in each period. Two laboratory methods were used to measure these parameters: Enzyme-linked immunosorbent assay (ELISA) for TGF-b1, albuminuria, cystatin C and colorimetric detection for proteinuria, BUN, creatinine. TGF-b1, creatinine, cystatin C, and BUN were measured in serum. Albumin and protein were measured in 24-hour urine.

Statistical analysis: Statistical analysis was done by SPSS (version 23, IBM, USA) software for Windows. All results were expressed as mean ± standard deviation (SD). Independent-sample t-test was used to compare the mean general characteristics of the participants. Statistical significance was analyzed by one-way ANOVA to compare the mean of the obtained data. After the one-way ANOVA test, Tukey post-hoc was used. The Kolmogorov-Smirnov test was used to analyze the normal distribution of data. P-values < 0.05 were considered significant

RESULTS

There was no significant difference in the results of anthropometric data such as height, weight, and body mass index between the control group and the diabetic group. Systolic and diastolic blood pressure of control individuals were compared with those of diabetics. Statistically, there was a significant difference in comparison of systolic and diastolic blood pressure between control and diabetic groups. (Correlation is significant at the 0.01 level (2-tailed)).

We also evaluated some important biomedical parameters (TGFb1, albumin, BUN, Cr, cystatin C, PU), in the serum sample of the control group and diabetics five times and four doses of squalene (The first day, fourteenth

day, the twenty-eighth day, the fifty-sixth day, the eighty-fourth day in 0 mg/day, 200 mg/day, 400mg/day and 600 mg/day). A significant difference was detected in the comparison of TGFb1 ($14^{th\ day/0}$, 14^{th} day in 200 mg/day, $28^{th/0}$, 28^{th} day in 200 mg/day, $56^{th/0}$,56^{th} day in 200 mg/day, $84^{th/0}$, 84^{th} day in 200 mg/day), AL ($28^{th\ day/0}$,28^{th} day/ in 200 mg/day, 56^{th} day/ in 200 and 400 mg/day, 84^{th} day in 400 and 600 mg/day), BUN ($28^{th/0}$, 28^{th} day in 200 mg/day, $56^{th/0}$,56^{th} day in 200 mg/day, $84^{th/0}$, 84^{th} day in 200 mg/day), Cr ($14^{th\ day/0}$, 14^{th} day in 200 mg/day, $28^{th/0}$, 28^{th} day in 200 mg/day, $56^{th/0}$,56^{th} day in 200 mg/day, 84^{th} day in 200, 400, 600 mg/day), cystatin C ($14^{th\ day/0}$, 14^{th} day in 200 mg/day, $28^{th/0}$, 28^{th} day in 200 mg/day, $56^{th/0}$,56^{th} day in 200 mg/day, $84^{th/0}$, 84^{th} day in 200 mg/day), PU ($14^{th\ day/0}$, 14^{th} day in 200 mg/day, $28^{th/0}$, 28^{th} day in 200 mg/day , 56^{th} day in 200, 400, 600 mg/day, 84^{th} day in 200, 400, 600 mg/day, Systolic ($14^{th\ day/0}$, 14^{th} day in 200 mg/day, $28^{th/0}$, 28^{th} day in 200 mg/day, $56^{th/0}$,56^{th} day in 200 mg/day, $84^{th/0}$, 84^{th} day in 200 mg/day), Diastolic ($14^{th\ day/0}$, 14^{th} day in 200 mg/day, $28^{th/0}$, 28^{th} day in 200, 400 mg/day, 56th/0,56th day in 200 mg/day, 84th/0, 84th day in 200 mg/day). There was a statistically significant difference (P value < 0.05) in the results of diabetic groups compared to the control group.

Figure 1 highlights the comparison of systolic and diastolic blood pressure in groups 1, 2, 3, 4, and 5. As shown in the diagram, both systolic and diastolic blood pressure increased in groups 2, 3, 4 and 5 compared to group 1. In the groups treated with different doses of squalene in groups 3, 4, and 5, on the recorded days, systolic and diastolic blood pressure decreased compared to group 2. Group 5, which consumed the highest amount of squalene (600 mg/daily), showed the lowest systolic and diastolic values as compared to the other diabetic groups that consumed squalene (200 mg/daily and 400 mg/daily). Similarly, group 4 (400 mg/daily) showed a lower systolic and diastolic value as compared to group 3 (200 mg/daily). From these results, it can be inferred that squalene functions effectively to reduce diastolic and systolic blood pressure.

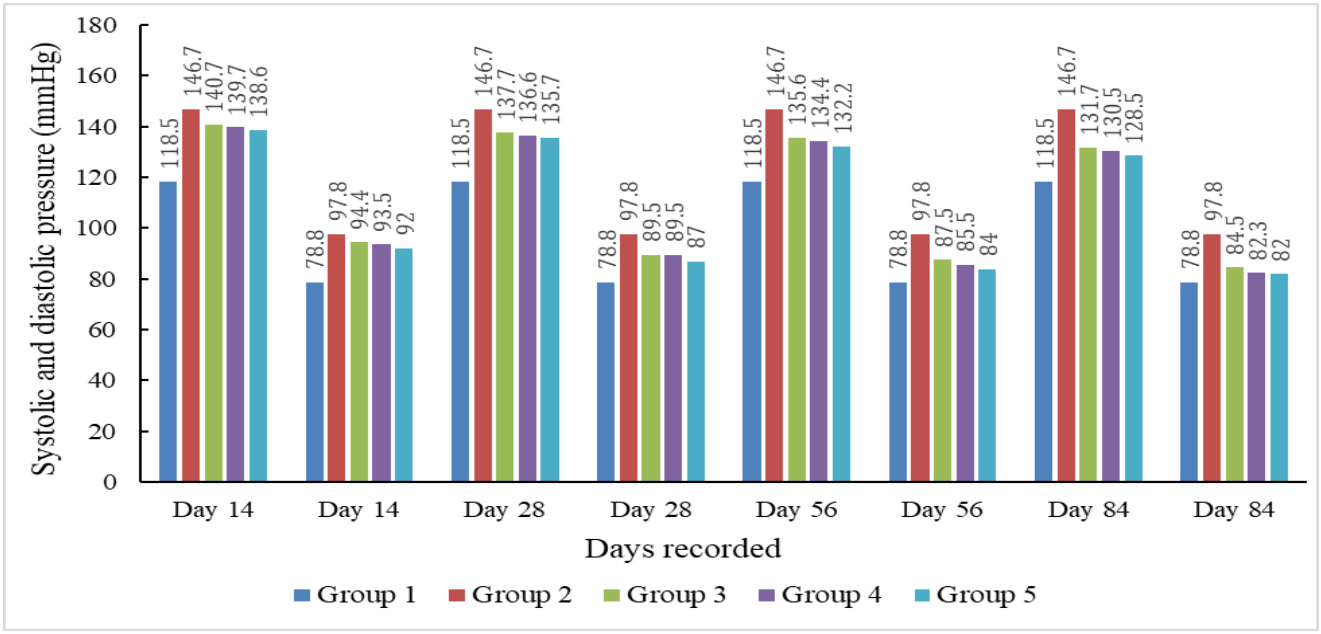


Figure 1: Comparison of systolic and diastolic blood pressure, respectively, recorded on different days in all groups. Group 1 represents the healthy group. Group 2 represents the diabetic control group. Group 3 represents the diabetic group that consumed 200 mg of squalene daily. Group 4 represents the diabetic group that consumed 400 mg of squalene daily. Finally, group 5 represents the diabetic group that consumed 600 mg of squalene daily. The first column cluster for each day recorded displays systolic blood pressure (mmHg); the second column shows diastolic blood pressure (mmHg).

From our results, we can see a reduction in proteinuria levels over the duration of the experiment. On day 0, the diabetic control who had not consumed squalene had an average proteinuria level of 19.02 mg/dl. Figure 2 depicts measurements collected for proteinuria throughout the duration of the 84-day study. These measurements were taken on days 14, 28, 56, and 84. On day 14, we began to observe a reduction in the level of proteinuria. This reduction showed a larger percentage difference with increased levels of squalene consumed. Group 3, which was administered 200 mg of squalene daily, had an average level of 18.04 mg/dl as their proteinuria level.

This was a 4.63% reduction from the diabetic control group. Group 4, which was administered 400 mg of squalene daily, had shown a 5.26% reduction. The average level of proteinuria for group 4 after 14 days had been 18.02 mg/dl. Group 5, which was administered 600 mg of squalene daily, exhibited the largest reduction in the percentage of proteinuria after 14 days, Group 5 had a 17.91 mg/dl level of proteinuria and a 5.84% reduction from the diabetic control group.

On day 28 (Figure 2), the trend of increased proteinuria level reduction in correlation to increased levels of squalene administered continued. Group 3 showed a 6.78% reduction from the diabetic control group, as their average proteinuria level was 17.73 mg/dl. Group 4 showed a 9.62% reduction from the diabetic control group, as their average proteinuria level was 17.19 mg/dl. Group 5 showed a 10.88% reduction, with their average proteinuria level being 16.95 mg/dl.

On day 56 (Figure 2), proteinuria levels continued to decrease with increased amounts of squalene administered. Group 3 showed a 10.41% reduction from the diabetic control group, as their average proteinuria level was 17.04 mg/dl. Group 4 showed a 15.72% reduction from the diabetic control group, as their average proteinuria level was 16.03 mg/dl. Group 5 showed a 21.03% reduction, with their average proteinuria level being 15.02 mg/dl.

On day 84 (Figure 2), final measurement recordings had been conducted. The observed trend of proteinuria reduction with increased amounts of squalene administered had also continued. Group 3 showed a 10.83% reduction from the diabetic control group, as their average proteinuria level was 16.96 mg/dl. Group 4 showed a 16.14% reduction from the diabetic control group, as their average proteinuria level was 15.95 mg/dl. Group 5 showed a 25.81% reduction, with their average proteinuria level being 14.11 mg/dl.

Throughout the course of the 84 days, proteinuria levels in the patient groups who consumed squalene showed a trend of reduction. The overall reduction as compared to the diabetic control group also showed a trend of increasing magnitude with elevated doses of squalene. Group 5, who consumed 600 mg of squalene daily, showed the highest percentage decrease of proteinuria consistently through each measured recording. This indicated to us that increased levels of squalene function more effectively and have a greater impact on reducing overall proteinuria levels. Further research may focus on the application of dosages higher than 600 mg, which may indicate if this reduction is maximized at a certain value of squalene. Throughout the 84 days, each interval shows consistent reduction in the overall average proteinuria level.

There was a key finding in proteinuria tracking and measurement from days 28 to 56. Group 5 (diabetic patients with 600 mg consumption of squalene) had initially shown a reduction in average proteinuria level from days 14-28 of 5.04%. From days 28 to 56, however, this percentage decreased by almost double. The percentage reduced from days 28 to 56 was 10.15%. This highlights an important portion related to doses, as a more effective dose can be seen past 56 days. In terms of treatment, long-term prescription of squalene can allow for maximized reduction in overall average proteinuria values.

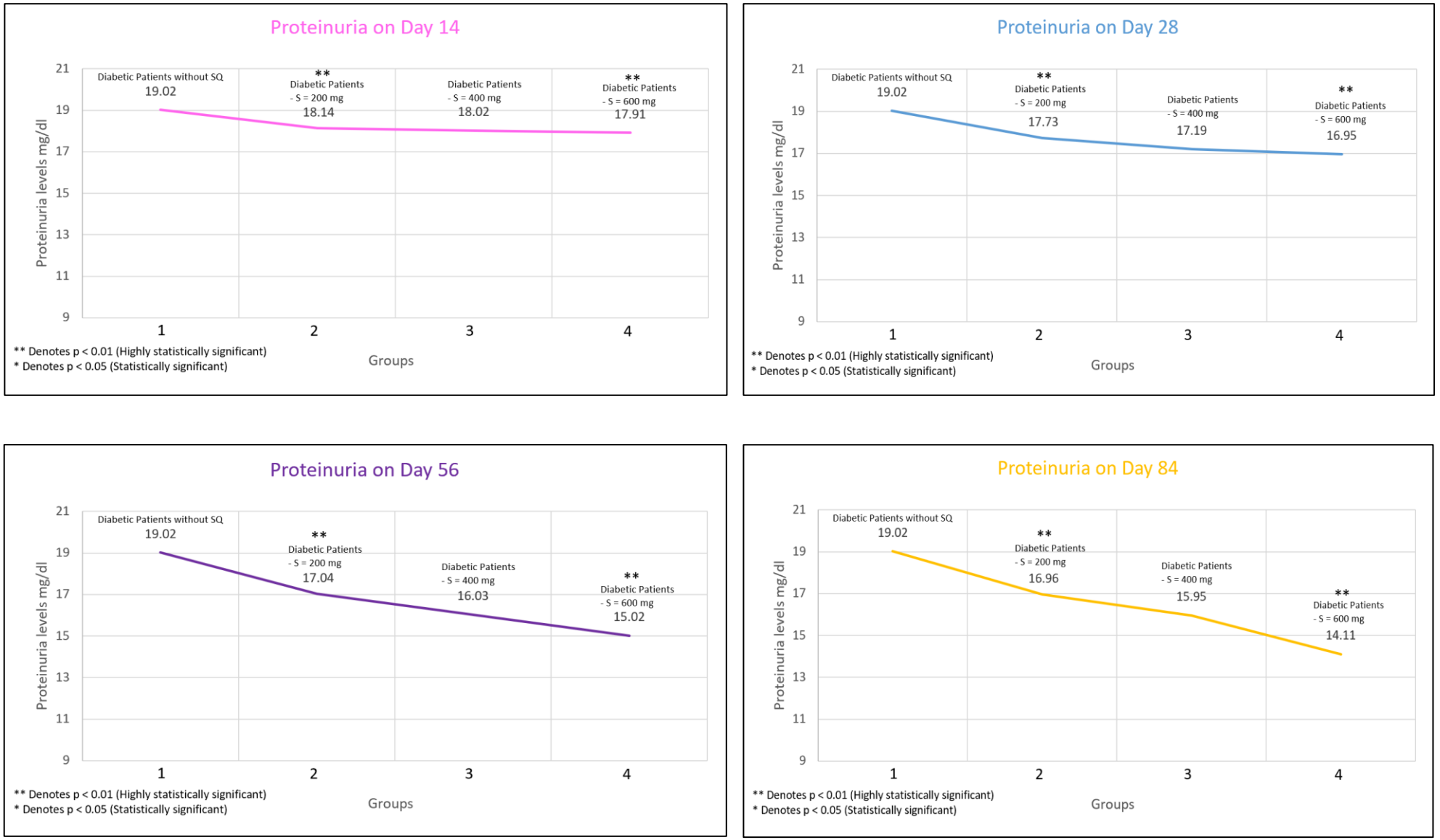


Figure 2. Graphical representations of data measurement recording date over the course of 84 days for proteinuria value.

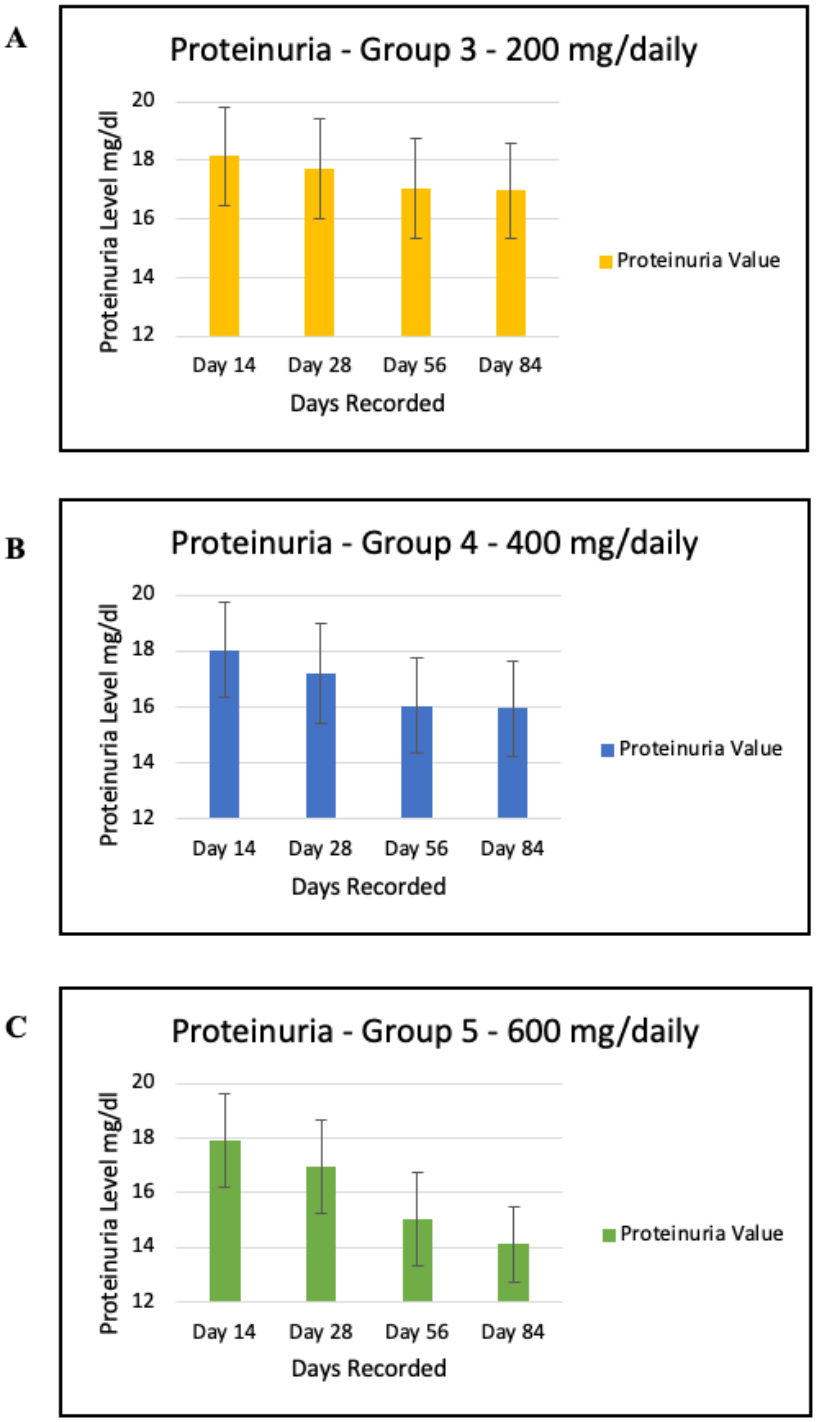


Figure 3 (A, B, C). Data are given as mean ± SD. Graphical representation for data measurements collected for individual group levels of proteinuria over the course of 84 days. These include groups 3, 4, and 5, which signify the diabetic groups which consumed squalene throughout the 84 days.

Figure 3 illustrates the data results collected for individual group levels of proteinuria over the course of 84 days. As such, group 3 shows the changes in the diabetic group that consumed 200 mg of squalene daily, group 4 shows the changes in the diabetic group that consumed 400 mg of squalene daily, and group 5 shows the changes in the diabetic group that consumed 600 mg of squalene daily.

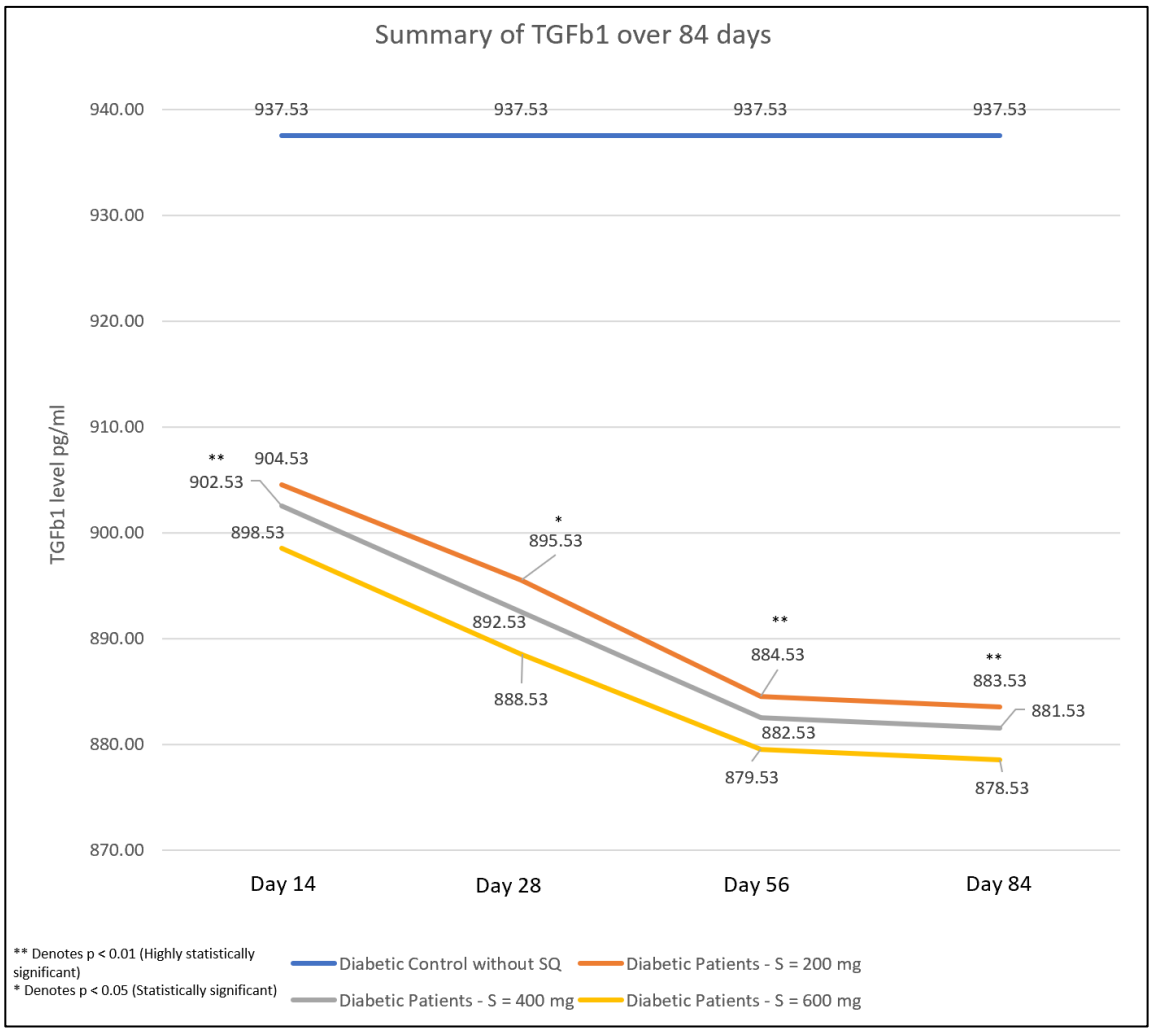


Figure 4. Graphical representation for data measurements collected for TGFbeta1 over 84 days.

Figure 4 depicts measurements collected for TGFbeta1 throughout the duration of the 84-day study. On day 0, the diabetic control group also had a TGFbeta1 level of 937.53. On day 14, TGFbeta1 levels in group 3 had a 3.52% reduction from the diabetic control group, with a value of 904.53. Group 4 had a 3.73% reduction from the diabetic control group, with a TGFbeta1 level of 902.53. Group 5 had a 4.16% reduction from the diabetic control group, with a TGFbeta1 level of 898.53. On day 28, TGFbeta1 levels in group 3 had a 4.48% reduction from the diabetic control group, with a value of 895.53. Group 4 had a 4.80% reduction from the diabetic control group, with a TGFbeta1 level of 892.53. Group 5 had a 5.23% reduction from the diabetic control group, with a TGFbeta1 level of 888.53. On day 56, TGFbeta1 levels in group 3 had a 5.65% reduction from the diabetic control group, with a value of 884.53 pg/ml. Group 4 had a 5.87% reduction from the diabetic control group, with a TGFbeta1 level of 882.53 pg/ml. Group 5 had a 6.19% reduction from the diabetic control group, with a TGFbeta1 level of 879.53 pg/ml. Finally, on day 84, final measurement recordings had been conducted. TGFbeta1 levels in group 3 had a 5.76% reduction from the diabetic control group, with a value of 883.53 pg/ml. Group 4 had a 5.97% reduction from the diabetic control group, with a TGFbeta1 level of 881.53 pg/ml. Group 5 had a 6.29% reduction from the diabetic control group, with a TGFbeta1 level of 878.53 pg/ml.

TGFbeta1 showed a minor reduction from days 14 to 28, a large reduction from days 28 to 56, and a minor reduction from days 56 to 84. Overall, however, it still showed a 6.29% decrease from initial testing. This illustrates that this parameter also showed a trend of reduction with increased squalene consumption.

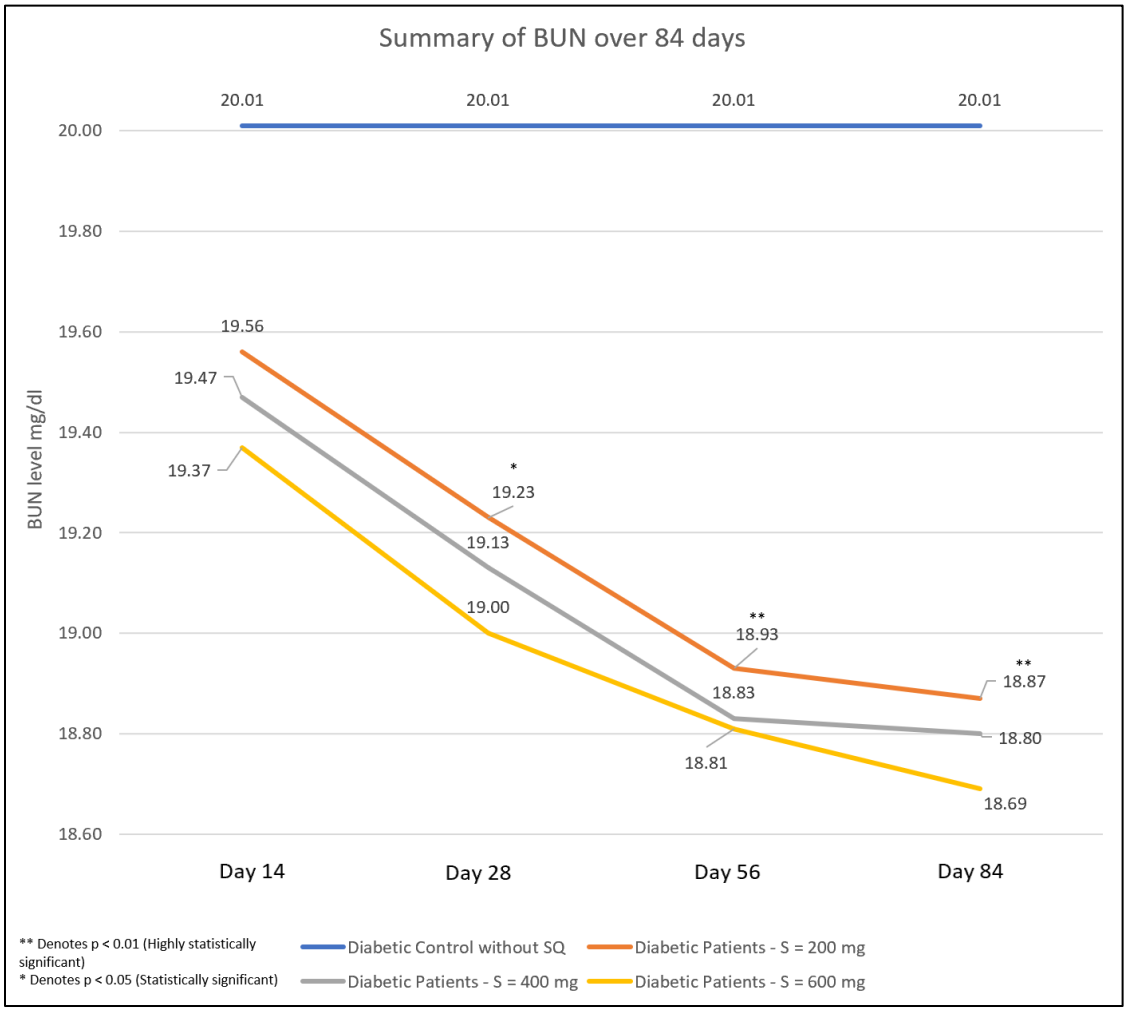


Figure 5: Graphical representation of data measurements collected for BUN (blood urea nitrogen) over 84 days.

Figure 5 depicts the measurements collected for BUN over the duration of the 84-day study. On day 0, the BUN level of the diabetic control group was 20.01 mg/dl. Figure 5 illustrates the recorded changes in BUN level in groups 1, 2, 3, 4, and 5 past day 0. On day 14, BUN levels in group 3 showed a 2.25% reduction from the diabetic control group, with a value of 19.56 mg/dl. Group 4 showed a 2.70% reduction from the diabetic control group, with a BUN value of 19.47 mg/dl. Group 5 showed a 3.20% reduction from the diabetic control group, with a BUN value of 19.37 mg/dl. On day 28, BUN levels in group 3 showed a 3.90% reduction from the diabetic control group, with a value of 19.23 mg/dl. Group 4 showed a 4.40% reduction from the diabetic control group, with a BUN value of 19.13 mg/dl. Group 5 showed a 5.05% reduction from the diabetic control group, with a BUN value of 19.00 mg/dl. On day 56, BUN levels in group 3 showed a 5.40% reduction from the diabetic control group, with a value of 18.93 mg/dl. Group 4 showed a 5.90% reduction from the diabetic control group, with a BUN value of 18.83 mg/dl. Group 5 showed a 6.00% reduction from the diabetic control group, with a BUN value of 18.80 mg/dl. Finally, on day 84, BUN levels in group 3 showed a 5.70% reduction from the diabetic control group, with a value of 18.87 mg/dl. Group 4 showed

a 6.05% reduction from the diabetic control group, with a BUN value of 18.80 mg/dl. Group 5 showed a 6.60% reduction from the diabetic control group, with a BUN value of 18.69 mg/dl.

The ratio of BUN to creatinine is commonly tested, also referred to as the BUN/Cr ratio. Previous studies have indicated that a high BUN/Cr ratio relates to the potential for fatality in patients with renal disease [29]. By measuring the average BUN level in each group, we were able to draw conclusions regarding its relation to squalene and proteinuria. We found that with daily squalene intake, the overall average BUN level in each group decreased as well. This may indicate that the BUN level may potentially be decreased due to either the consumption of squalene or the reduction in proteinuria. Previous research has illustrated the relation between BUN and proteinuria, which both showed a decrease with the treatment of vitamin E in mice [10]. In order to understand more about the dynamics of these trends, future studies may focus on a thorough analysis of the biochemical effect that squalene has on BUN.

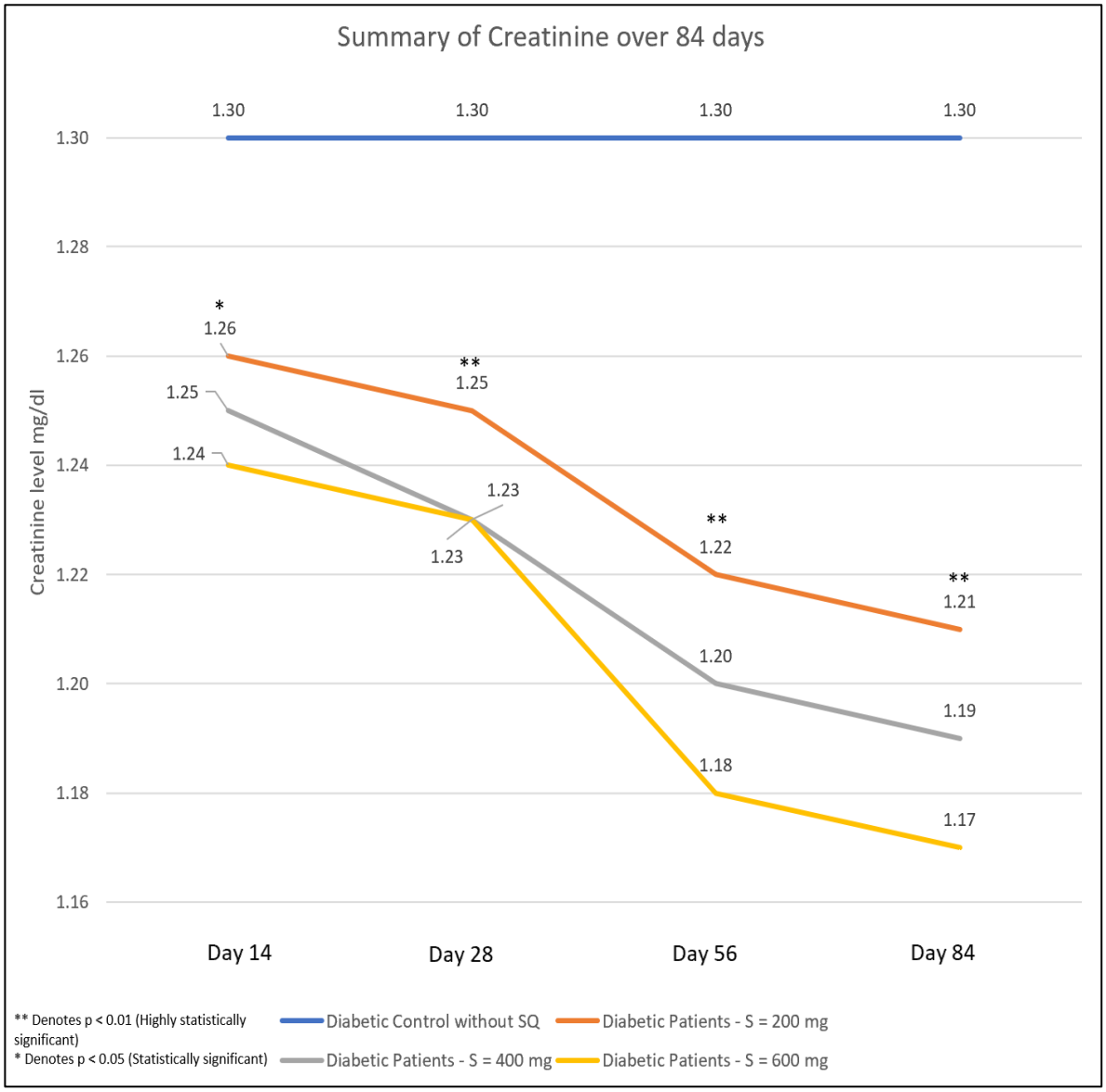


Figure 6. Graphical representation of data measurements collected for creatinine over 84 days.

Figure 6 depicts the measurements collected for creatinine over the duration of the 84-day study. On day 14, Cr levels in group 3 had a 3.08% reduction from the diabetic control group, with a value of 1.26 mg/dl. Group 4 had a 3.85% reduction from the diabetic control group, with a Cr level of 1.25 mg/dl. Group 5 had a 4.62% reduction from the diabetic control group, with a Cr level of 1.24 mg/dl. On day 28, Cr levels in group 3 had a 3.85% reduction from the diabetic control group, with a value of 1.25 mg/dl. Group 4 had a 5.38% reduction from the diabetic control group, with a Cr level of 1.23 mg/dl. Group 5 had a 5.38% reduction from the diabetic control group, with a Cr level of 1.23 mg/dl. On day 56, Cr levels in group 3 had a 6.15% reduction from the diabetic control group, with a value of 1.22 mg/dl. Group 4 had a 7.69% reduction from the diabetic control group, with a Cr level of 1.20 mg/dl. Group 5 had a 9.23% reduction from the diabetic control group, with a Cr level of 1.18 mg/dl. Finally, on day 84, Cr levels in group 3 had a 6.92% reduction from the diabetic control group, with a value of 1.21 mg/dl. Group 4 had an 8.46% reduction from the diabetic control group, with a Cr level of 1.19 mg/dl. Group 5 had a 10.00% reduction from the diabetic control group, with a Cr level of 1.17 mg/dl.

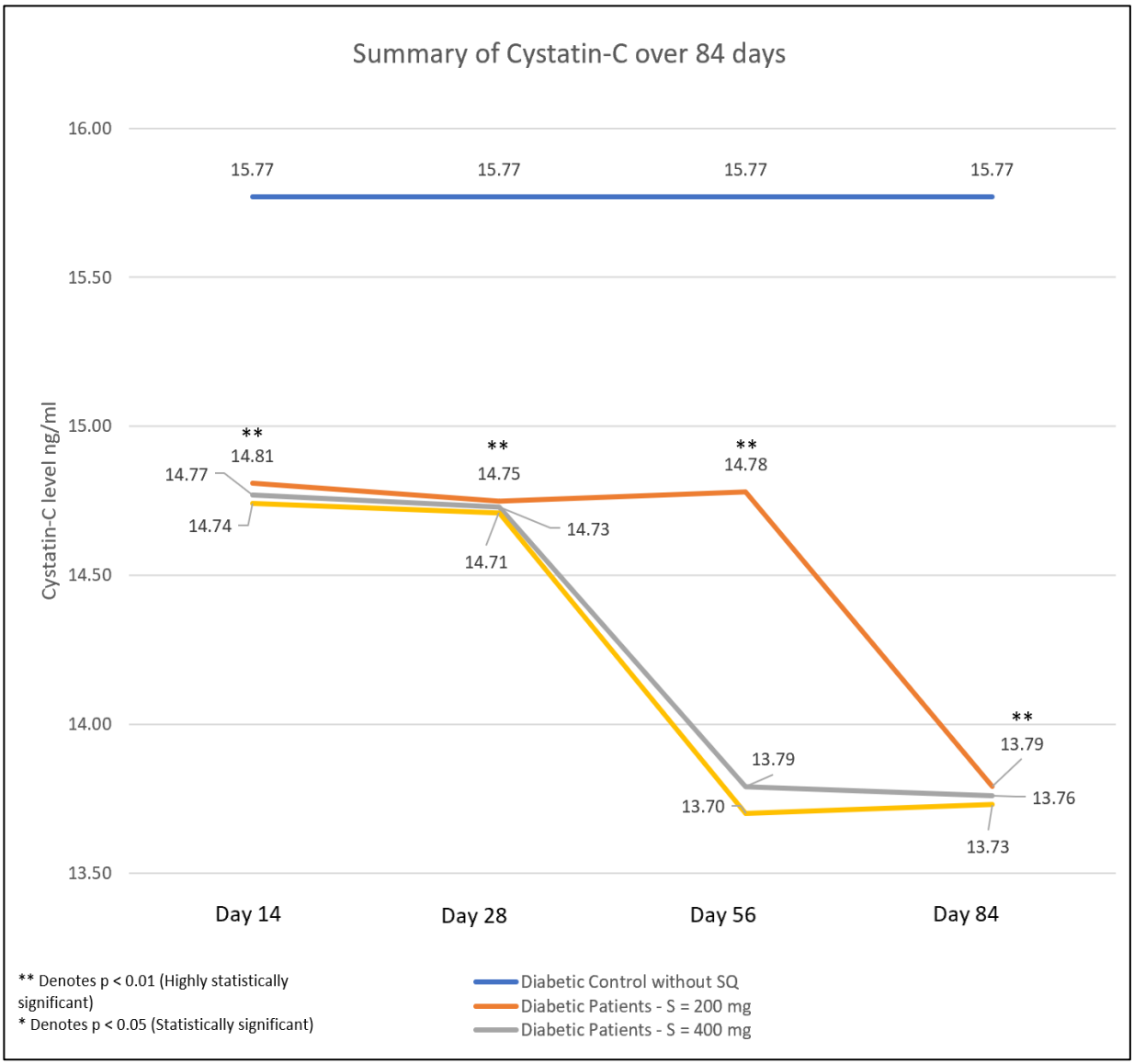


Figure 7. Graphical representation for data measurements collected for cystatin C over 84 days.

Creatinine is a type of byproduct which is released through the kidney. It relates to our overall study as adequate filtration and release of creatinine may indicate kidney disease. Throughout the 84 days, creatinine levels showed a similar trend of reduction with consumption of squalene. Group 5, which consumed the largest dosage of proteinuria, showed the greatest reduction in creatinine levels. Higher doses of squalene intake correlated with a higher impact of creatinine reduction. The largest percent reduction occurred from days 28 to 56. Previous studies have shown the effectiveness of the creatinine index as a preliminary screening tool for proteinuria [30]. The results of this study solidify the relationship between creatinine and proteinuria, as both of these parameters exhibited a similar trend of reduction in average value.

Figure 7 depicts the measurements collected for cystatin C over the duration of the 84-day study. On day 0, the diabetic control group had a cystatin C value of 15.77 ng/ml. On day 14, cystatin C levels in group 3 had a 6.09% reduction from the diabetic control group, with a value of 14.81 ng/ml. Group 4 had a 6.34% reduction from the diabetic control group, with a cystatin C level of 14.77 ng/ml. Group 5 had a 6.53% reduction from the diabetic control group, with a cystatin C level of 14.74 ng/ml. On day 28, cystatin C levels in group 3 had a 6.47% reduction from the diabetic control group, with a value of 14.75 ng/ml. Group 4 had a 6.59% reduction from the diabetic control group, with a cystatin C level of 14.73 ng/ml. Group 5 had a 6.72% reduction from the diabetic control group, with a cystatin C level of 14.71 ng/ml. On day 56, cystatin C levels in group 3 had a 6.28% reduction from the diabetic control group, with a value of 14.78 ng/ml. Group 4 had a 12.56% reduction from the diabetic control group, with a cystatin C level of 13.79 ng/ml. Group 5 had a 13.13% reduction from the diabetic control group, with a cystatin C level of 13.70 ng/ml. Finally, on day 84, cystatin C levels in group 3 had a 12.56% reduction from the diabetic control group, with a value of 13.79 ng/ml. Group 4 had a 12.75% reduction from the diabetic control group, with a cystatin C level of 13.76 ng/ml. Group 5 had a 12.94% reduction from the diabetic control group, with a cystatin C level of 13.73 ng/ml.

Interestingly, cystatin C did not show consistency in trend throughout the duration of the study. Unlike the previous parameters which showed a continuous trend of reduction, cystatin C exhibited a change in trend on day 56. From the recordings and measurements taken on days 14 and 28, cystatin C showed a reduction with squalene consumed. Like the previous parameters, higher doses of squalene intake correlated with a higher reduction in cystatin C. However, this changed dramatically on day 56. Group 3 (diabetic patients with 200 mg intake of squalene daily) showed an increase in overall average cystatin C levels. Groups 4 (diabetic patients with 400 mg intake of squalene daily) and 5 (diabetic patients with 600 mg intake of squalene daily) both showed a dramatic reduction in cystatin C levels at this point. From days 14 to 28, group 4 had a reduction of 0.25%. This jumped to a reduction of 5.97% from days 14 to 28. Similarly, group 5 had a reduction of 0.19% from days 14 to 28. This jumped to a reduction of 6.41% from days 28 to 56. This indicates that the most effective dosages would be either 400 mg or 600 mg, with 600 mg showing the most reduction. However, this was altered even more so from days 56 to 84. On day 84, the overall average cystatin C level for group 5 (diabetic patients with 600 mg intake of squalene daily) increased by 0.19%. This indicates that if 600 mg of squalene is

prescribed for dosage, the maximum it should be used for is 56 days. After passing this time, cystatin C values may show an increase with this specific dosage. On day 84 as well, group 4 showed a minor reduction in percent as compared to the drop from days 28 to 56. This indicates that cystatin C reduction with squalene may also be most effective up until day 56. Further research and data collection on the 400 mg dose may allow for a better understanding of this. Group 3 (diabetic patients with 200 mg intake of squalene daily), however, showed a major reduction at this point. Although this group had a slight increase from days 28 to 56, this dropped by 6.28% from days 56 to 84. This indicates that for a dosage of 200 mg of squalene daily, it may be more effective to prescribe for a total of 84 days, as this allows for the rebound of reduction. Throughout the 84 days, the lowest cystatin C value was 600 mg of squalene at day 56. Further research may allow for an understanding of how time impacts cystatin C with intake of squalene.

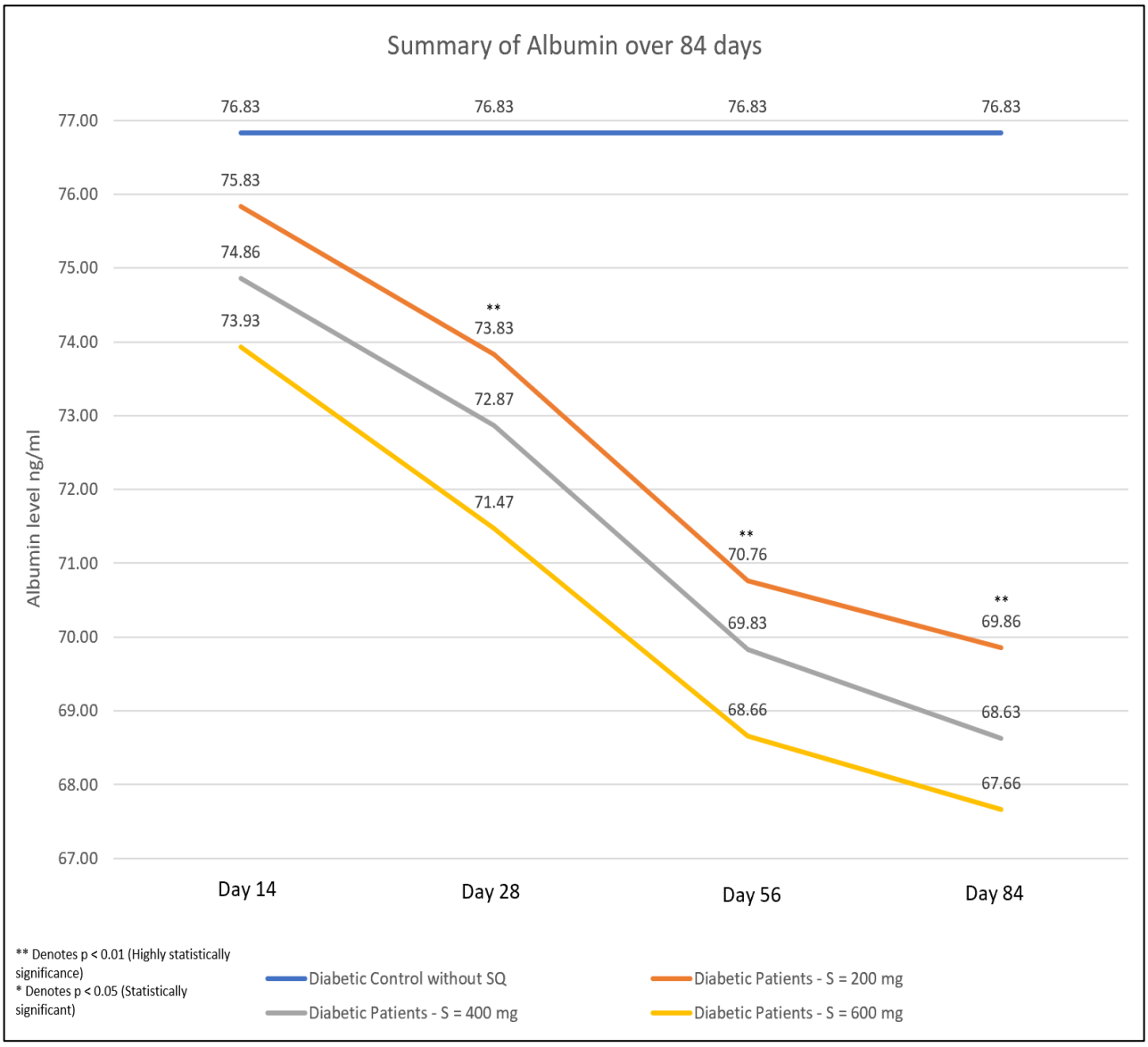


Figure 8. Graphical representation of data measurements collected for albumin over 84 days.

Figure 8 depicts the measurements of Albumin collected throughout the duration of the 84-day study. On day 0, the diabetic control group had an AL level of 76.83 ng/ml. Figure 8 shows the changes in AL levels in groups 1, 2, 3, 4, and 5 past day 0. On day 14, AL levels in group 3 showed a 1.30% reduction from the diabetic control group, with a value of 75.83 ng/ml. Group 4 showed a 2.56% reduction from the diabetic control group, with an average AL level of 74.86 ng/ml. Group 5 showed a 3.77% reduction from the diabetic control group, with an average AL level of 73.93 ng/ml. On day 28, AL levels in group 3 showed a 3.90% reduction from the diabetic control group, with a value of 73.83 ng/ml. Group 4 showed a 5.15% reduction from the diabetic control group, with an average AL level of 72.87 ng/ml. Group 5 showed a 6.99% reduction from the diabetic control group, with an average AL level of 71.46 ng/ml. On day 56, AL levels in group 3 showed a 7.90% reduction from the diabetic control group, with a value of 70.76 ng/ml. Group 4 showed a 9.11% reduction from the diabetic control group, with an average AL level of 69.83 ng/ml. Group 5 showed a 10.63% reduction from the diabetic control group, with an average AL level of 68.66 ng/ml. Finally, on day 84, AL levels in group 3 showed a 9.07% reduction from the diabetic control group, with a value of 69.86 ng/ml. Group 4 showed a 10.67% reduction from the diabetic control group, with an average AL level of 68.63 ng/ml. Group 5 showed a 11.94% reduction from the diabetic control group, with an average AL level of 67.66 ng/ml.

Albumin is also a type of protein. As it is synthesized in the liver, albumin is commonly used in blood testing to identify potential health problems in the liver and kidney. In our study, albumin also exhibited a similar trend in reduction, as it decreased by an overall of 11.94% over 84 days.

DISCUSSION

Proteinuria is a commonly used indicator for renal disease [16]. Therefore, a higher proteinuria value found in the kidney correlates to an increased likelihood of renal disease. Renal disease presents a public health issue due to the severity and prevalence arising in the human population [17]. Amaranth oil is composed largely of squalene, which indicates this may be a key biotic compound causing the reduction of proteinuria levels [18-21]. Squalene is a biotic compound found naturally in shark liver, amaranth oil, and olive oil [22-27]. The benefits of squalene have been an area of interest to researchers due to recent findings regarding the impact squalene has on human health. One such benefit is the reducing effect it has on cancer. It was determined in a study that squalene prevented tumor growth through inhibition of the promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) in mice [28]. In terms of classification, squalene, as a bioactive compound, belongs to the triterpene class [30]. Current treatments for proteinuria include medication intake, change in diet, and change in exercise. For individuals with high blood pressure or diabetes, focus is on anti-hypertensive treatments. In order to determine if squalene functioned as the proteinuria reducing agent in amaranth oil, we focused our study on intake of various different dosages of serum squalene in diabetic patients. These patients were studied over a course of 84 days and were tested with an interval of 14 days. This was composed of a healthy control group, a diabetic control group, a diabetic group with 200 mg/daily consumption of squalene, a diabetic group with 400 mg/daily consumption of squalene, and a diabetic group with 600 mg/daily consumption of squalene. By doing so, this enabled us to observe any trends in relation to squalene intake and average proteinuria levels. The application of different dosages also allowed us to understand the threshold of highest reduction levels of proteinuria.

There were key findings throughout the study highlighted in our results. On day 28, the proteinuria value for group 5 (diabetic patients who consumed 600 mg squalene/daily) showed a reduction of 10.88%. However, on day 56, group 5's proteinuria value reduction was 21.03%. This indicates an increase in

reduction rate by 93.29%. Overall, the final and highest proteinuria reduction was in group 5 on day 84, with a reduction of 25.81% from the diabetic control group. Another key finding was identified in measurement of cystatin C. On day 28, group 5 had an average cystatin level reduction by 6.72%. However, on day 56, this reduction increased to 13.13% (shown in Figure 8). This also indicated an increase in reduction rate change by 95.38%. However, after this point, the reduction in cystatin C for group 5 decreased to 12.94%.

According to FFC's definition of functional food as well as the quantum theory of Functional Food Science [31], it is extremely important to find the exact amount of bioactive compounds in the process of developing functional food products. One of the most important steps to create functional foods involves establishing an appropriate dosage for the identified bioactive compound [32]. Dosage is important as it is defined by the therapeutic range in which a compound may exhibit positive effects in users. Biological activity has been acknowledged with bioactive compounds that have a positive effect, but negative effects are also a form of bioactivity [33]. This includes adverse effects such as toxicity, allergenicity, and mutagenicity, which are usually dependent on the dose and bioavailability of a given substance. In fact, evidence exists regarding the risk of consuming an excessive amount of healthy substances such as antioxidants, omega 3, and soy isoflavones [34]. In addition, according to the Functional Food Center's definition, creation, classification, and regulation of functional food, products should not only be researched through preclinical and clinical studies by using biomarkers. To make ideal functional food products they should be done through epidemiological and after market studies to come up with the ideal functional food product [35].

It has been shown that squalene, with its antioxidant and anti-inflammatory properties, can protect various cells in the body against oxidants and ROS [36]. Squalene can protect skin cells from the sun by preventing lipid peroxidation [37]. By in vitro studying on renal carcinoma cell (RCC) line, Rajamani et al. showed that squalene can induce its anti-inflammatory properties by reducing the nuclear factor kappa B (NF-κB). Blood urea nitrogen (BUN) and creatinine were biochemical factors that were studied in the blood of rats treated with squalene and squalene with potassium bromate (KBrO3). In their study, there was no significant difference in the reduction of BUN and creatinine in rats treated with squalene alone, but a significant difference in these two parameters was observed between control rats and rats treated with both squalene and KBrO3 [38].

CONCLUSION

In addition to the statistical significance found in systolic and diastolic blood pressure, there was also observed statistical significance in changes in BUN, albumin, Proteinuria, cystatin C, TGFbeta1, and creatinine levels. As illustrated in figures 1-8, we can visualize these changes through graphical representation. All of the associated health indicative parameters we tested alongside proteinuria count also showed a trend of overall reduction throughout the duration of 84 days. These values vary based on the dosage of squalene consumed as well as the time elapsed from initial prescription. Based on these results, it may be assumed that squalene functions effectively to reduce all of these parameters as well as overall proteinuria level. The mechanism behind how squalene reduces proteinuria levels remains unclear. This study helps advance the field by providing results indicating the significant reduction that intake of serum squalene alone had on overall proteinuria level. Squalene can be investigated in deeper analysis to understand the mechanisms and interactions

that allow it to do so. The parameters we studied also showed similar trends of reduction with increased amounts of squalene consumed. With cystatin C, the trend is harder to interpret due to fluctuations in overall value within each group tested. By extending the duration of the experiment, an in-depth analysis may show the overall effect that squalene has on this parameter.

These results are only preliminary, and some limitations may reside with the sample size and duration of investigation. Future clinical, epidemiological, as well as aftermarket studies of squalene related products may strengthen the findings of this study by using a larger number of diabetic patients within each group. Another area which can be focused on is the extension of the study over a longer time, as this can allow for better understanding of the trend that certain parameters exhibited. This could be done with aftermarket studies by using squalene for a longer time than we investigated in this study (84 days).

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List of abbreviations: ACE, angiotensin converting enzyme; BUN, blood urea nitrogen; CR, creatinine; DM, diabetes mellitus; HbA1c, glycated hemoglobin; T2DM, type 2 diabetes; TGF-B, transforming growth factor b1.

Authors' contributions: All authors contributed to this work. HM contributed to the selection of volunteers to participate in the study, doing the experimental work and conducting the clinical trial. MRA and ASM participated in data collection. SP contributed to analysis of the results. AS participated in the manuscript writing and drawing the graphs of the results. AB participated in the editing and formatting. DM discussed idea of squalene as a contributing factor to manage proteinuria with HM and participated in the study design and supervised the project. The authors read and approved the final version before its submission.

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