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Research Article

Impact of Treating Hyperuricemic NAFLD Patients with Allopurinol on Cytokeratin 18: A Pilot Study

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Abstract

Introduction: Elevated Uric acid strongly associates nonalcoholic fatty liver disease, in the same time there is a growing body of evidence pointing to the role of cytokeratin 18 as a NAFLD serological marker. Thus, we aimed by this study to evaluate the impact of the uric acid lowering drug Allopurinol in treatment of hyperuricemic non-alcoholic fatty liver disease patients on cytokeratin 18.

Methods: 31 hyperuricemic ultrasound diagnosed non-alcoholic fatty liver disease patients of a mean age of 44.3 ± 11 years were enrolled into the study and grouped into; group A (14 patients) who received starch based tablets and group B (17 patients) who received allopurinol (100 - 300 mg), for 3 months for both groups. Cytokeratin 18, adiponectin, and fatty liver grade by ultrasound were measured at baseline and at the end of the study.

Results: The study showed a significant decline in cytokeratin 18 within group B ($p = 0.006$), yet, neither of the 2 groups showed change in adiponectin or fatty liver grade by US.

Conclusion: Thus, we concluded that Allopurinol treatment for hyperuricemic non-alcoholic fatty liver disease patients may stop the disease progression as mirrored by cytokeratin 18 decline, which warrants further studies on a large scale.

Keywords: *Uric Acid; NAFLD; Allopurinol; CK 18; Adiponectin; Ultrasound; Fatty Liver Grade*

Abbreviations

NAFLD: Non-Alcoholic Fatty Liver Disease; ROS: Reactive Oxygen Species; NASH: Non-Alcoholic Steatohepatitis; UA: Uric Acid; US: Ultrasound; CK: Cytokeratin; TC: Total Cholesterol; HDL-C: High Density Lipoprotein Cholesterol; LDL-C: Low Density Lipoprotein Cholesterol; TG: Triglycerides; FL: Fatty Liver; ELISA: Enzyme-Linked Immunosorbent Assays; XO: Xanthine Oxidase

Introduction

Non-alcoholic fatty liver disease (NAFLD), is a common cause of chronic liver disease that represents a current major health problem [1].

NAFLD is characterized by a 'Multiple-hit' pathogenesis. The initial hit is insulin resistance leading to an increased uptake and synthesis of free fatty acids resulting in simple fatty liver. The second hits involves oxidative stress from reactive oxygen species (ROS) and an altered production of adipokines. These pathways probably play the most prominent role in the progression of NAFLD to nonalcoholic steatohepatitis (NASH) and can be potentially involved in promoting the negative impact of superimposed insults ultimately leading to progressive liver disease [2].

Multiple studies show that when uric acid (UA) enters the cells via specific transporters plays a pro-inflammatory role where it acts as a pro-oxidant, inducing the release of inflammatory mediators and growth factors, moreover, it is shown to contribute to lipoprotein oxidation and inflammation [3].

This oxidative stress directly caused by UA may partially explain why elevation of UA significantly increased the risk of NAFLD, also generation of UA, is accompanied by generation of ROS which might act as the ''second hit'' that induces NAFLD development [4].

Although liver biopsy is still considered the gold standard in diagnosis and the only reliable mean for distinguishing NASH from simple steatosis and for grading and staging the disease yet, non-invasive investigations, such as liver functions and imaging techniques, offer considerable promise in their ability to differentiate simple steatosis from significant fibrosis and to stage liver fibrosis in 80 - 90% of cases, where ultrasonography (US) is currently the preferred method for screening suspected NAFLD as it demonstrates hepatomegaly and diffuse increase in echogenicity of the liver parenchyma [5].

Lately, there has been increasing interest in the role of cytokeratin (CK) proteins during apoptosis. Though the significance of CK degradation during apoptosis is unclear, it has been suggested that caspase (aspartate-specific cysteine proteases) cleavage of the CK proteins possibly facilitates the formation of apoptotic bodies and magnifies the apoptotic signal when activated [6].

Obesity-linked down-regulation of adiponectin may mechanistically play a role in insulin resistance and diabetes. Clinically, adiponectin has been reported to inversely correlate with obesity and NAFLD. Naturally, NAFLD patients have significantly lower plasma adiponectin levels and insulin resistance [7].

Aim of the Study

Aim of this pilot study was to have an insight into the impact of the UA lowering drug allopurinol on hepatic condition as reflected by CK18, when used in hyperuricemic NAFLD patients.

Patients and Methods

This is a prospective, randomized, placebo-controlled, single blinded pilot study conducted on NAFLD patients with hyperuricemia.

Patients

Over a 6 months recruitment period, 134 patients referring the liver outpatient clinic at the National Research Centre were screened for eligibility, where 31 patients were enrolled as per the following inclusion/exclusion criteria, a) inclusion: i- both genders, ii- age ≥ 25 years old, iii- elevated serum uric acid (> 7 mg/dL in men, and > 6 mg/dL in women), iv- treatment free of statins, fenofibrates, liver support and allopurinol for the past 3 months, v- sonographically detected fatty change of the liver, b) exclusion: i- viral hepatitis, ii- alcohol consumption more than 40 g per week for the past 12 months, iii- diabetes mellitus, iv- hypertension, v- autoimmune liver disease, vi- malignancy of any nature, vii-any systemic failure (cardiovascular, renal or respiratory), viii- major psychiatric illness, ix- pregnancy or lactation.

Finally 31 patients were enrolled and randomly assigned into two groups:

1. **Group A (Placebo):** Fourteen patients that received starch-based placebo tablets.
2. **Group B (Treatment):** Seventeen patients received allopurinol 100 mgs initially increased to 300 mgs gradually if needed [8].

Allopurinol was supplied as film coated tablets containing either 100 or 300 mg of allopurinol under the trade name of Zyloric® manufactured by GlaxoSmithKline El Salam City, Cairo, Egypt under license from the GlaxoSmithKline group of companies.

Follow up visits

Both groups were followed up on monthly basis for 3 months period for ruling out adverse events and ensuring compliance, together with thorough clinical examination, and blood tests for liver transaminases (ALT-AST), lipid profile (total cholesterol TC, high density lipoprotein cholesterol HDL-C, low density lipoprotein cholesterol LDL-C, and triglycerides TG), serum UA. At baseline and last visits they were subjected to laboratory work up comprising; CK 18, and adiponectin together with abdominal US.

Laboratory work

Serum preparation

Blood samples were collected without using an anticoagulant and were allowed to clot for 30 minutes at 25°C. Serum samples were isolated by centrifugation at 4000 rpm and stored at -80°C until assayed.

Enzyme-linked immunosorbent assays (ELISA) were used for the measurements of serum Cytokeratin 18 (Glory Science Co., Ltd, TX, USA) and serum Adiponectin (Assaypro LLC, St. Charles, MO 63304) levels.

Human Cytokeratin 18-M30 (CK18-M30) ELISA Kit

Method

CK 18 was determined using commercial Enzyme-linked immunosorbent assays (ELISA) kit manufactured by Glory Science Co., Ltd 2400 Veterans Blvd. Suite 16 - 101,Del Rio, TX 78840, USA.

Principle of test

The kit used a double-antibody sandwich ELISA to assay the level of CK-18 in the sample, purified human CK-18 was adopted to coat microtiter plate, solid phase antibody was made, then CK-18-M30 (CK18-M30) was added to monoclonal antibody enzyme well which was pre-coated with human CK-18-M30 (CK18-M30) monoclonal antibody, incubation; then, CK-18-M30 (CK18-M30) antibodies labeled with biotin was added, and combined with streptavidin-HRP to form immune complex; then incubation was carried out and washed again to remove the uncombined enzyme.

Then chromogen Solution A, B, was added, the color of the liquid changes into the blue, the reaction is terminated by the addition of a stop solution and the colour change is measured at a wavelength of 450 nm. The concentration of CK-18 in the sample is then determined by comparing the optical density of the samples to the standard curve.

Human adiponectin ELISA kit

Method

Adiponectin level was determined using commercial ELISA kit manufactured by Assay pro LLC, St. Charles, MO 63304. (catalog no. EA2500-1).

UA

Determination of UA was done by enzymatic colorimetric method [9].

Reference values are less than 6 mg/dl for women and less than 7 mg/dl for men.

Transaminases (AST and ALT)

AST reference values are less than 40 u/l for men and less than 32 u/l for women, while ALT reference values are less than 38 u/L for men and less than 31 u/l for women.

Determination of AST and ALT was done by kinetic method [10].

Lipid profile (Chol, TG, HDL-C, LDL-C)

Reference values:

- Chol: Less than 200 mg/dl
- TG: Less than 150 mg/dl
- HDL-C: Not less than 35 mg/dl for women and 45 mg/dL for men.
- LDL-C: Less than 150 mg/dl.

Cholesterol, triglyceride, HDL and LDL-cholesterol levels are determined by enzymatic colorimetric method [11].

US

NAFLD was classified into 3 grades:

1. **Grade I:** Minimal diffuse increase in the fine echoes. Liver appears bright compared to the cortex of the kidney. Normal visualization of diaphragm and intrahepatic vessel borders.
2. **Grade II:** Moderate diffuse increase in the fine echoes. Slightly impaired visualization of the intrahepatic vessels and diaphragm.
3. **Grade III:** Marked increase in the fine echoes. Poor or no visualization of intrahepatic vessels and diaphragm and poor penetration of the posterior, segment of the right lobe of the liver [12].

Statistical methods

- Data management and analysis were performed using Statistical Package for Social Sciences (SPSS) vs. 21.
- Numerical data were summarized using means and standard deviations while Categorical data were summarized as percentages.
- The Chi-squared test was used to compare between the study groups with respect to categorical data.
- Paired t-test was used to compare between the study groups with respect to parametric data.
- P-values < 0.05 were considered significant.

Ethical considerations

The study protocol was revised and approved by the research ethics committee for experimental and clinical studies at National Research Center-Cairo-Egypt. Prior to participation each of the enrolled patients was educated about the study protocol and signed the written informed consent. Each of the eligible patients was randomly assigned by block randomization to either group A or group B.

Results

A total of 134 patients were assessed for eligibility, and thirty one NAFLD patients suffering from hyperuricemia fulfilled the criteria and were enrolled in the study and randomized into group A (placebo) patients and group B (treatment).

Baseline characteristics

The demographic, biochemical and radiological data of both groups as shown in table 1 demonstrates 14 patients in group A, 8 males and 6 females with an age range of 26 to 58 years (mean ± SD 43.9 ± 9.5 years), while group B consisted of 17 patients,13 males and 4 females with an age range of 25 to 57 years (mean ± SD 44.3 ± 11 years) where no significant difference was shown between the two groups. Group B showed significantly higher transaminases mean value as compared to group A yet it was still within the upper range of normal. In group A, 13 patients were found to be grade II fatty liver (FL) and one patient to be grade III FL, while in group B 9 patients were of grade I FL, 6 patients grade II FL and 2 patients grade III FL, with significantly higher percentage of patients harboring higher FL grades within group B.

	Group A N = 14	Group B N = 17	Total	P value
Age (Mean ± SD)	43.9 ± 9.5	44.7 ± 12.3	44.3 ± 11.0	0.848①
Gender: N (%)				0.224②
Male	8 (57.1)	13 (76.5)	21 (67.7)	
Female	6 (42.9)	4 (23.5)	10 (32.3)	
BMI: N (%)				
≤ 25	0(%)	3 (17.6%)	3 (9.7%)	
>25 - 30	3 (21.4%)	5 (29.4%)	8 (25.8%)	0.474①
> 30	11 (78.6%)	9 (52.9%)	20 (64.5%)	
Mean ± SD	35.1 ± 7.2	33.0 ± 8.6	34.0 ± 7.9	
Uric Acid mg/dl				0.217①
Mean (±SD)	7.2 (0.7±)	7.5 (0.9±)	7.4 (0.8±)	
Cholesterol mg/dl				0.139①
Mean (±SD)	181.4 (21.1±)	200.9 (44.1±)	192.1 (36.5±)	
TG mg/dl				0.518①
Mean (±SD)	177.2 (102.1±)	157.2 (67.8±)	166.2 (80.1±)	
HDL mg/dl				0.173①
Mean (±SD)	38.9 (3.4±)	36.6 (5.0±)	37.6 (4.4±)	
LDL mg/dl				0.904①
Mean (±SD)	135.1 (31.2)	136.5 (29.3)	135.9 (29.6)	
GPT u/l				
Mean (±SD)	17.6 (±6.8)	30.8 (±13.1)	24.8 (±12.5)	0.002*①
GOT u/l				
Mean (±SD)	18.1 (±5.4)	25.3 (±10.2)	20.0 (±9.0)	0.023*①
CK18 ug/l				0.308①
Mean (±SD)	32.1 (±8.2)	35.0 (±7.1)	33.7 (±7.6)	
Adiponectin *µg/ml*				0.319①
Mean (±SD)	10.2 (±2.)	9.5(±1.8)	9.8(±1.9)	
FL size			9 (20.0)	
Mean (SD)	16.6 (0.9)	16.1 (1.3)	19 (61.3)	0.210①
			3 (9.7)	
FL Grades: N (%)				
One	0	9 (52.9)		
Two	13 (92.9)	6 (35.3)		0.001*②
Three	1 (7.1)	2 (11.8)		

Table 1: *Baseline demographic, clinical, biochemical and radiological characteristics of both study groups.*

Statistical test: ① = Student t-test, p-value > 0.05: non-significant

Statistical test: ② = X^2 Test, p-value > 0.05: non-significant

NS: Non-Significant

**Statistically significant*

We compared different study parameters within both groups as regards baseline and end of study values.

UA

As shown in figure 1, the mean value of UA dropped significantly in group B from a mean value of 7.5 (±0.9) mg/dl at baseline, to a mean value of 5 (±0.9) mg/dl at the end of the study p < 0.001, while group A showed a borderline significant drop from a mean value of 7.2 (±0.7) mg/dl at baseline to a mean value of 6.6 (±1.4) mg/dl at the end of the study p = 0.05.

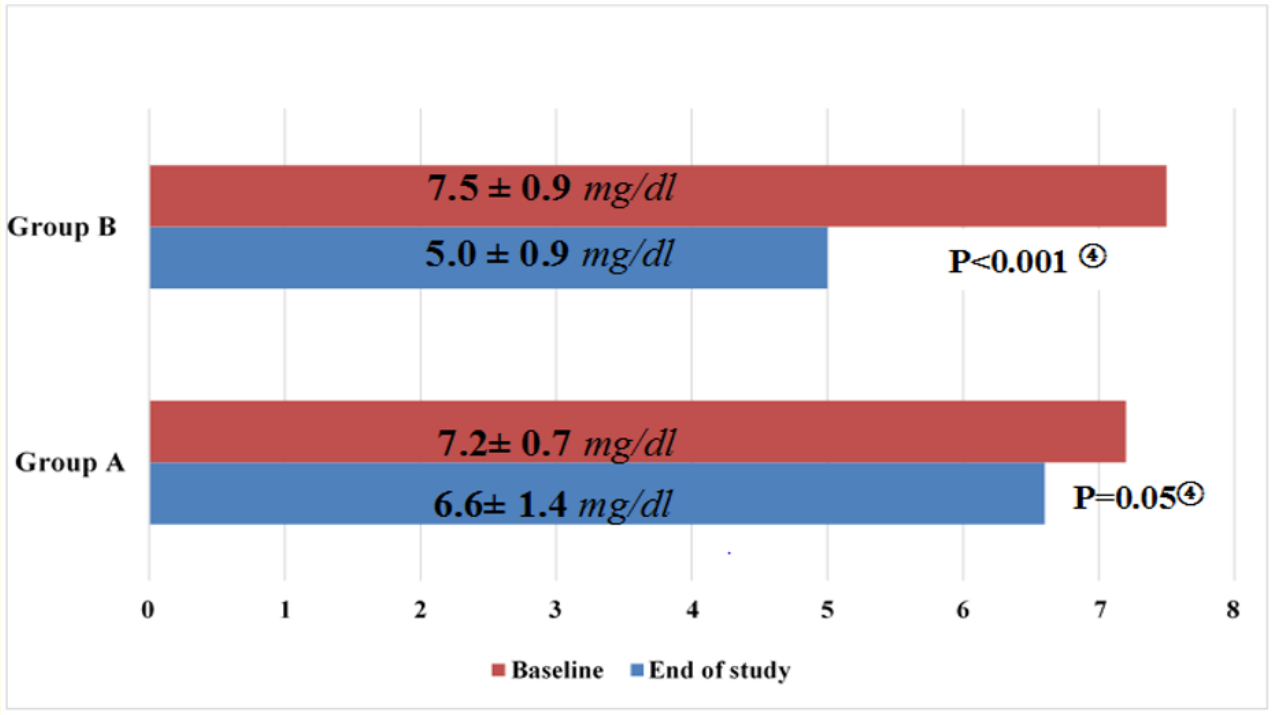

Figure 1: *Comparing between uric acid change at baseline and end of study within both groups.*

④ = Student T test

CK 18

As shown in figure 2 CK 18 mean serum level declined significantly in group B from baseline to end of the study (35.0 ± 7.1 µg/l to 28.8 ± 7.8 µg/l respectively) while there was no significant difference between baseline and end of study values (32.1 ± 8.2 µg/l and 28.8 ± 7.8 µg/l respectively) in group A.

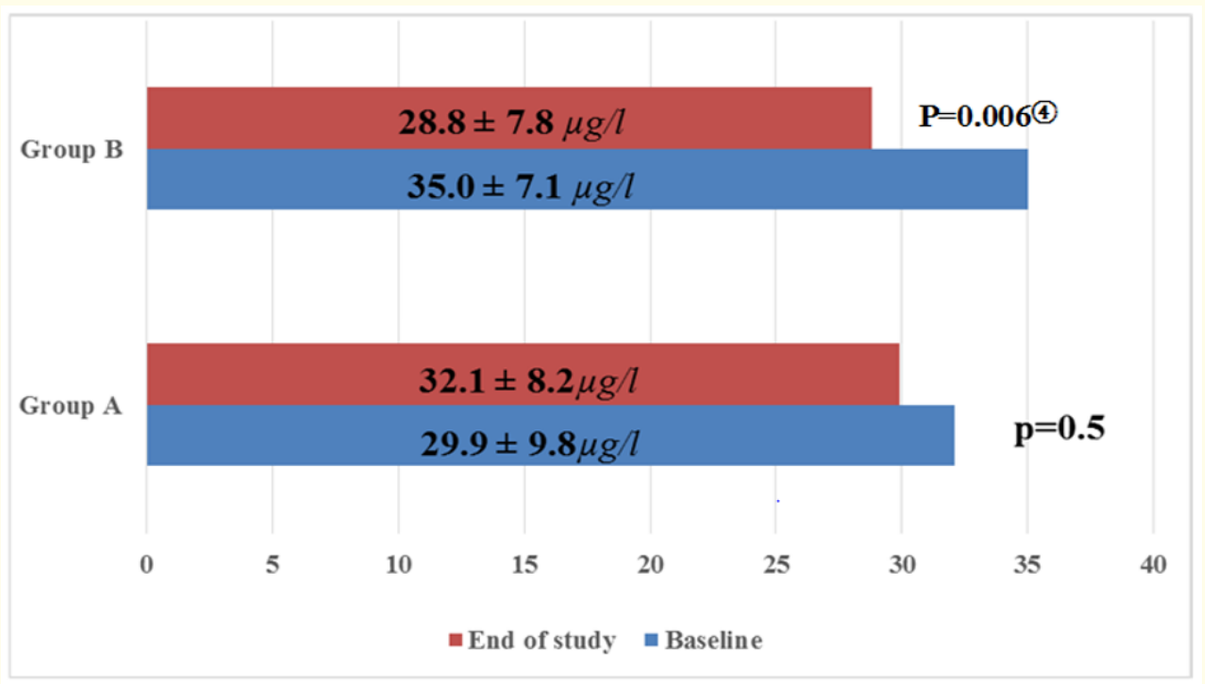

Figure 2: *Comparing between cytokeratin 18 at baseline and end of study within both groups.*

④ = Student T test

Adiponectin

Neither groups showed any significant change in adiponectin values at baseline as compared to end of study as shown in figure 3, where group A showed a mean value of 10.2 ± 2 µg/ml at baseline and 10.1 ± 2.4 µg/ml at end of study p = 0.5, while group B showed a mean value of 9.5 ± 1.8 µg/ml at baseline and 10.3 ± 2.3 µg/ml at end of study p < 0.006.

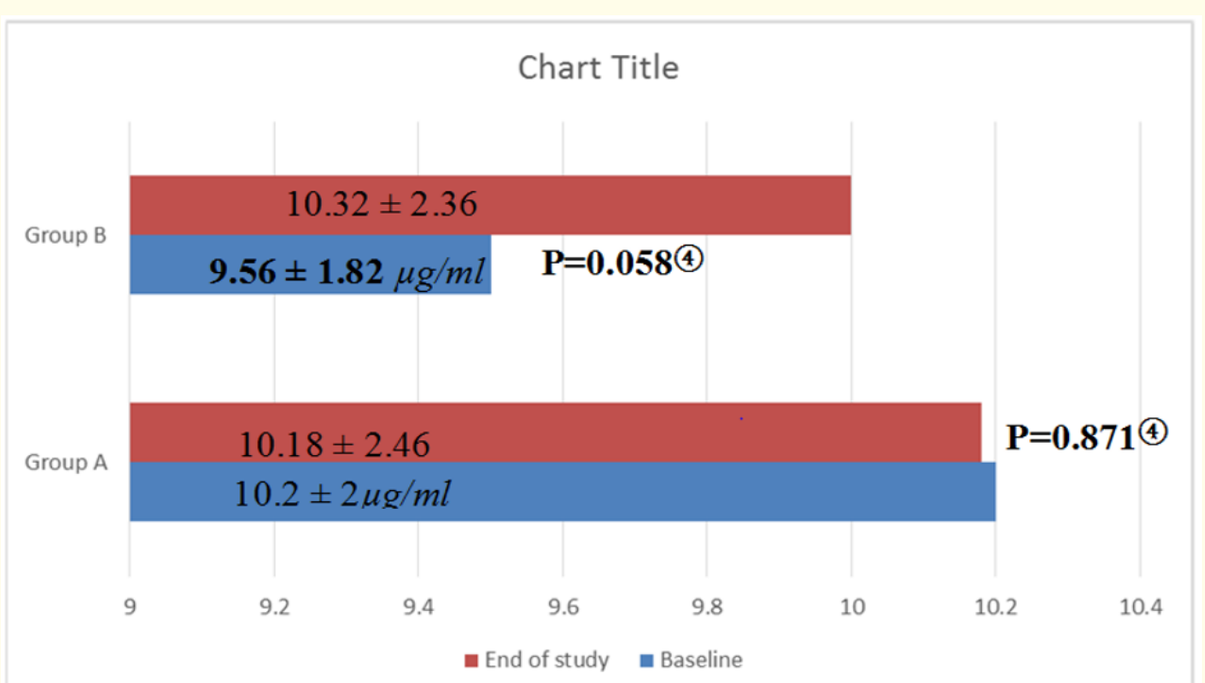

Figure 3: *Comparing between Adeponectin at baseline and end of study within both groups.*

④ = Student T test

FL grades

As mentioned above, we classified our patients on basis of abdominal US findings into either of 3 grades (I-II-III) of FL, after Mahaling., *et al.* classification [12].

Accordingly, we compared the percentage of patients within the different grades at baseline to that post treatment, where 0%, 92.9% and 7.1% of group A patients were grade I, II and III respectively, at baseline, that insignificantly changed to be 14.3%, 85.7% and 0% respectively at the end of the study p = 0.1. Likewise, 52.9%, 35.3% of group B patients were grade I, II respectively, at baseline that insignificantly changed to be 41.2%, 47.1% respectively and 2% were grade III both at baseline and at the end of the study p = 0.3.

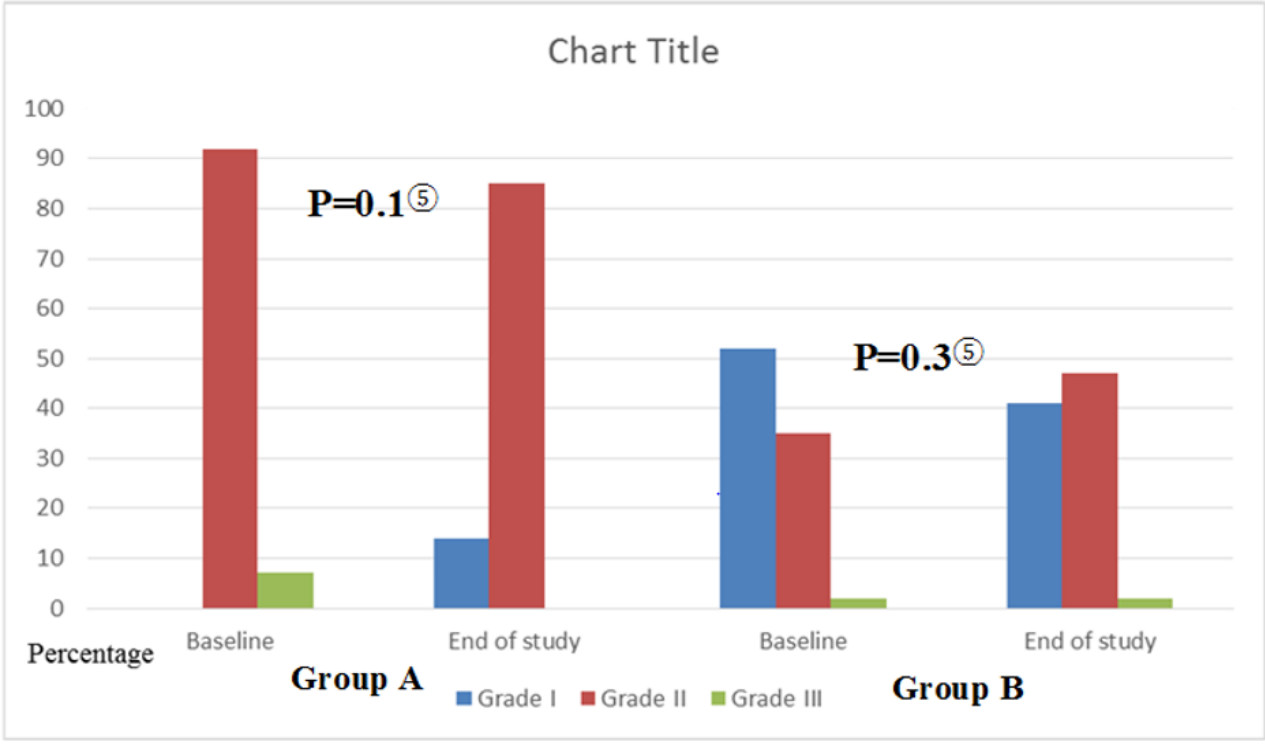

Figure 4: *Changes in fatty liver grades from baseline to end of study within both groups.*

⑤ = *McNemar test*

Discussion

NAFLD is the result of hepatic fat accumulation in patients without a history of excessive alcohol consumption, its prevalence has been rising rapidly making it the most common chronic liver disease in the developed world today [13], it is strongly associated with obesity, insulin resistance, hypertension and dyslipidemia, it is regarded as the liver manifestation of the metabolic syndrome [14].

Hyperuricemia as well, has been linked to the metabolic syndrome and cardiovascular diseases, as it increases the risk of endothelial dysfunction, carotid atherosclerosis which may contribute to vascular inflammation and artery damage, thereby increasing the risk for cardiovascular diseases [15]. An early study by Lonardo., *et al.* in 2002 described an association between US diagnosed NAFLD and serum UA levels in a small case-control study of Italian patients [16]. Later on Jeffrey C., *et al.* in 2013 confirmed the association between high

serum UA levels and NAFLD in 10,732 non-diabetic adults who participated in the National Health and Nutrition Examination Survey 1988 - 1994 in the USA, the research group defined sex specific UA quartiles as: ≤ 5.2, 5.3 - 6.0, 6.1 - 6.9 and > 6.9 mg/dL for men and ≤ 3.7, 3.8 - 4.5, 4.6 - 5.3 and > 5.3 mg/dL for women where they found that the prevalence of any grade of NAFLD was highest in the 4th quartile [3]. Chengfu Xu., *et al.* in 2015 found that the expression and activity of XO are significantly increased in cellular and mouse models of NAFLD and that inhibiting XO activity by allopurinol significantly decreased hepatic steatosis in high fructose diet-fed mice which led them to suggest XO as a novel therapeutic target for patients with this disease [17] as lowering UA with allopurinol might help in improving liver enzymes levels in patients with NAFLD [18]. Thus, the primary objective of this prospective, randomized, single blinded, placebo -controlled pilot study was to study the impact of xanthine oxidase (XO) inhibitor UA lowering drug allopurinol when administered to hyperuricemic NAFLD patients, while our secondary objective is to evaluate this impact through the hepatic apoptosis marker CK18. Hepatic apoptosis plays a crucial role in hepatic fibrogenesis and hence liver disease progression, as engulfment of apoptotic bodies by hepatic stellate cells stimulates the fibrogenic activity of the later [19] while DNA from apoptotic hepatocytes acts as an important mediator of hepatic stellate cell activation [20]. In hepatocytes of patients with NAFLD, regardless of the triggering stimuli, the apoptotic process tends to converge at the level of the mitochondria resulting in permeabilization of the mitochondrial outer membrane and release of multiple proteins from the mitochondrial intermembrane space into the cytosol. The result of this process is the activation of the effector caspases (mainly caspase-3), which cleave different substrates inside the cell including CK-18 the major intermediate filament protein in the liver resulting in apoptosis - which explained a role of CK 18 as a diagnostic marker in NAFLD [21]. Accordingly, CK 18 has been extensively studied as a biomarker for NAFLD, its prognosis and severity. Wieckowska and his co-workers in 2006 carried on a small pilot study using a specific immune ELISA assay which showed that CK18 markedly associated NASH and correlated with the presence of fibrosis [22] which was supported later on in a diverse population of patients [23] and in an independent population of morbid obese subjects [24], while subsequently other groups have reported similar results [25,26] Interestingly, CK 18 was not just studied as a marker associating NASH, but also as a mirror reflecting changes of the hepatic status, where decline in CK18 level correlated to improvement in liver histology in terms of steatosis grade and hepatocellular ballooning [27] as well as to improvement in NAS score as in patients with an improved NAS, there was a significant improvement in the and the mean CK-18 levels declined significantly with improvement in NAS score and showed a static pattern in patients with a static NAS [28] moreover, CK 18 serum levels decreased dramatically 6 months following bariatric surgery in a study carried on 65 patients [24]. This study exploited this particular aspect of CK18 as a NAFLD prognostic marker to evaluate the impact of allopurinol. Our study showed a significant decline in serum CK18 level after 3 months of allopurinol treatment, on contrary to patients who were administered a placebo, which might point to a role of allopurinol in improving hepatic condition in NAFLD as mirrored by the decrease in hepatocytes apoptotic process. Nevertheless, this CK18 decline was not associated with an improvement of FL grades as graded by US, where the percentage of patients at the 3 FL grades (I-II-III) did not change significantly from baseline to end of study. In this context it is worth noting that US has its limitations in diagnosing and to a greater extent in follow up of NAFLD patients where some studies pointed to the substantial intra- and inter-observer variability [29,30] indicating the operator dependence as a drawback of the technique. Additionally, the qualitative nature of the current four-point grading system is too simplistic to account for small alterations in steatosis severity on follow-up which questions the adequacy of US for evaluating patients with NAFLD after therapeutic intervention, where computerized quantitative analysis methods for US may be able to overcome these limitations, but they require further clinical validation [31]. Thus, using US in follow up of NAFLD post therapeutic intervention could be a limitation to our study hindering early detection of steatosis regression post treatment, specially that the time point of evaluation three months post treatment could be relatively early.

We were much concerned as well about studying the effect of allopurinol on adiponectin as UA is taken up in adipocytes by an organic anion transporter where it induces oxidative stress via activation of NADPH oxidase, generating oxidized lipids and inflammatory mediators such as monocyte chemoattractant protein-1 (MCP-1) which leads to adiponectin synthesis inhibition [32]. A significant linear relation between high UA and low adiponectin serum levels was shown in a Petta., *et al*'s study carried on 166 NAFLD patients [18].

Allopurinol was found to attenuate the local inflammatory response in the visceral fat, reduce the expression of inflammatory cytokines, and enhance circulating levels of adiponectin in association with an improvement in insulin resistance in hyperuricemic pound mouse [33]. This pilot study showed a potential role for allopurinol in improving hepatic condition in NAFLD patients as reflected by significant decrease in CK18, but the study has some limitations, first all our patients had liver transaminases within the upper limit of normal on contrary to all studies evaluating CK18 as a marker for NAFLD, second owing to the nature of the study being a pilot study, we were limited by a small sample size which tied us from adjusting for different demographic, clinical and biochemical parameters and the short follow up duration and finally depending on US for NAFLD follow up post therapeutically. Thus we regard this study as a suitable brick to build on further studies on a large population of NAFLD for attaining a perspective about the feasibility of incorporating allopurinol into the panel of therapies of NAFLD, likewise we do highly appreciate extensively studying the reliability of CK18 as a NAFLD biomarker.

Conclusion

Thus, we concluded that Allopurinol treatment for hyperuricemic non-alcoholic fatty liver disease patients may stop the disease progression as mirrored by cytokeratin 18 decline, which warrants further studies on a large scale.

Conflict of Interest

None.

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