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Research Article

Biomarkers of iron status in allopurinol-treated renal stone patients

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Abstract

Limited evidence exists on the effect of xanthine oxidase inhibitors in nephrolithiasis patients on iron status markers, beyond their effects on urate. The aim of this study was to investigate whether allopurinol therapy was associated with a significant impact on parameters related to iron status, in patients with renal stones. Allopurinol treatment was associated with a nonsignificant decline in serum uric acid. There were no significant differences in serum levels of transferrin and ferritin after treatment with allopurinol compared to pre-treatment levels. A non-significant fall in serum levels of haptoglobin was registered. The drug was associated with a significant rise in serum iron levels. Serum uric acid and iron did not show a significant correlation with any parameter in the study. Allopurinol exerted an overall non-significant effect on iron metabolism in nephrolithiasis patients, save for serum iron, this entails lack of untoward effects in populations with-iron related conditions.

Keywords

Allopurinol, ferritin, hyperuricemia, iron, nephrolithiasis

Introduction

Urolithiasis is among the commonest urinary tract ailments, the past decades witnessed increasing in its prevalence and incidence in all age groups and in both genders, mainly in industrial nations, with an estimated global prevalence of 20 percent (Silva and Maciel 2016; Assadi and Moghtaderi 2017). Renal stones based on calcium comprise 80% of all kidney stones and of these, 80% are calcium oxalate (CaOx) in structure (Finkielstein and Goldfarb 2006), while urate stones comprise 10% and thus are rated as the commonest after CaOx and phosphate stones (Ferrari and Bonny 2004).

Uric acid (UA), the final catabolic product of purine metabolic pathway in humans, is a primary etiological factor in kidney urate and CaOx stones (Feig et al. 2013). Additionally, the elevated level of UA, hyperuricemia (HU), is a recognized factor implicated in many conditions like obesity, non-fatty liver disease, diabetes, hypertension and chronic kidney disease (CKD) (Karis et al. 2014). Biosynthesis of UA is a two-step process whose catalyst is the enzyme xanthine oxidase (XO) (Benn et al. 2018). Xanthine oxidoreductase (XOR) is a multifunctional iron-molybdenum-sulfur -containing enzyme, existing in 2 interconverting forms: Xanthine dehydrogenase (XDH) and xanthine oxidase (XO) (Guthikonda et al. 2003). The sole enzyme system in humans responsible for UA production is XO (Guthikonda et al. 2003; George and Struthers 2009).

Structurally related to purines, allopurinol is an inhibitor of XO diminishing UA endogenous synthesis; it

is among the commonest urate lowering therapy (ULT) modalities used (Yasui et al. 2001; Aung et al. 2017). It reduces stone formation in hyperuricosuric subjects suffering from recurrence in UA stones and /or gout (Ferrari and Bonny 2004). By blocking UA biosynthesis, it reduces the possibility of nucleation of UA/monosodium urate crystals into CaOx calculi (Heilberg and Schor 2006). Whether urate urinary excretion is within normal limits or the patient is hyperuricosuric, allopurinol proved effective in reducing CaOx calculi recurrence in either case (Yasui et al. 2001).

Urate nephrolithiasis or urolithiasis starts with precipitation of urate crystals in medullary interstitial regions of kidney; then calculi whose major composition is urate are formed; alternatively, urate crystals serve as nuclei to initiate CaOx precipitation (Heilberg and Schor 2006; Benn et al. 2018). Nephrolithiasis usually appears when factors like high serum UA concentration and low urine pH co-exist (Skoczyńska et al. 2020). However, urate stones are increasing in prevalence probably due to other factors like obesity, metabolic syndrome and consuming diets rich in purines (Abate et al. 2004). Occasionally, measures like enhanced fluid intake and urine alkalinization fail to resolve urate nephropathy (Skoczyńska et al. 2020), some patients with kidney stones may fail the dietary measures or require lowering of urate burden (e.g. those with gout, calcium-based kidney stones or urate nephropathy), it is these patients who are candidates for XO inhibitors (XOIs) (Pacher et al. 2006; Ngo and Assimos 2007). ULT indications span a range of gout-related conditions, in addition to concomitant conditions like UA urolithiasis and CKD (Khanna et al. 2012; Aung et al. 2017).

Despite major health concern that iron deficiency constitutes, its magnitude in different settings remains unidentified, and hemoglobin, routinely used to assess anemia is an underperforming anemia biomarker (Suchdev et al. 2017). Interestingly, anemic condition and hyperuricemia share some underlying co-morbidities like cardiovascular disease (CVD) and CKD and thus may actually be correlated conditions (Eun et al. 2019). Current evidence indicates that status of iron and its metabolism may be an etiological factor in elevated UA levels (Ghio et al. 2005; Mainous et al. 2011; Flais et al. 2017). Thus elevated iron may confound or contribute to HU and resultant gout, thus controlling it hypothetically may prove beneficial for urate-related pathologies (Alenezi et al. 2020). While correlations between UA and iron status markers exist and are studied earlier (Liu et al. 2013; Beyl Jr et al. 2016; Yang et al. 2017), however, confounding factors limit their relevance. Such correlations may theoretically be influenced by XOI therapy. Limited literature surveying this claim in urolithiasis patients maintained on allopurinol exists. The relationship between iron status biomarkers and allopurinol therapy in kidney stone patients has not been addressed previously thoroughly, if any, and exploring this area, about which limited evidence exists in literature, to the best of our knowledge, may thus be warranted.

Given these intricate and at best incoherent correlations between iron, uric acid and XO, we hypothesize that XO inhibition via allopurinol may have implications related to iron metabolism, extending beyond its effects on urate, such that the magnitude of drug effect would warrant continued use of the drug in this patient category or prove otherwise. The present study is conducted with the aim of investigating the effect of XO inhibition with allopurinol on the specific parameters of the iron metabolism/ status in urolithiasis patients.

Materials and methods

Study design and subjects

Open-label, prospective design was adopted in the current study. The study included 15 subjects (6 females, 9 males). Eligible patients were those presenting with either urate or CaOx stones for which allopurinol was prescribed and continued for 6 months at a dose of 300 mg/ day. Written informed consent was obtained from patients who agreed to participate in the study. Demographic and anthropometric data, including age, height, weight, gender as well as other relevant patient information were gathered by filling in a questionnaire for each subject and information was collected by direct interview with study subjects.

Those with underlying renal pathology other than urolithiasis, those with urinary tract infection, acute or chronic liver conditions, CVD, conditions associated with chronic inflammation, anemia or any other chronic clinical condition, as well as pregnant and lactating women, were excluded. Those with past or current use of ULTs, those on cyclophosphamide, azathioprine, 6-mercaptopurine or thiazide diuretics were excluded as well. Those who performed regular blood donation, were taking iron supplements, or anti-inflammatory agents were screened for and excluded as well.

Patients were instructed to fast overnight and attend the clinic in the morning. Blood was obtained by standard venipuncture procedure, and then left to clot in plain tubes at room temperature. Serum separation was carried out by centrifuging the sample tubes. Serum obtained was stored frozen at -20 °C pending analysis. Anthropometric (height and weight) and other data were obtained and samples were taken at 2 occasions: at commencing the study (time zero), and at completing the 6-month period of continued treatment (i.e., before and after allopurinol therapy).

Estimation of serum uric acid

Serum UA was determined by enzymatic colorimetric method (PAP Uricase procedure). Uricase enzyme brings about oxidation of UA thereby producing hydrogen peroxide, carbon dioxide, and allantoin. Amino-antipyrine and dichlorohydroxybenzene sulfonate (DHBS) (a chromogen) will react with the hydrogen peroxide, thus generating a colored substance (quinoneimine). The

absorbance of the analyte is then quantitated colorimetrically at a wavelength of 505 nm (Gochman and Schmitz 1971; Islam et al. 2018).

Estimation of serum iron

Serum iron (SI) was determined colorimetrically using Ferene method. Transferrin-bound iron is dissociated in acidic medium, reduction of ferric iron to ferrous iron occurs via ascorbic acid. Resulting Fe^{+2} then forms a colored complex with Ferene (3 -(2-Pyridyl) -5, -6-difuryl-1, -2, 4-triazine-disulfonate). The resultant chromogen absorbance is measured at 600 nm (Smith et al. 1984).

Estimation of serum haptoglobin, transferrin and ferritin

Sandwich ELISA technique (Schwab 2011) was used for quantification of serum haptoglobin (Hp) (Elabscience kit, cat#E-EL-H6078), serum transferrin (TF) (Elabscience kit, cat#E-ELH6028) and serum ferritin (FT) (Elabscience kit, #E-EL-H0168). The procedure was carried out according to manufacturer's (Elabscience, Houston, Texas, USA) instructions.

Statistical analysis

The R software (version 4.0.5) (R Core Team 2021) was used for statistical analysis. In addition to base R functionality, R package dplyr (Wickham et al. 2021) was used for managing/accessing tabulated data as well as calculating descriptive statistics; ggplot (Wickham 2016) R package was used for visualizing data and constructing graphs. For data in the current study, mean, standard deviation (SD) and standard error of the mean (SEM) were calculated. When data paired differences were normally distributed, paired samples *t*-test was used to compare means of baseline and post-treatment readings; otherwise, Wilcoxon signed ranks test for paired data was used; *P*-value < 0.05 was considered significant. Additionally, Pearson's correlation was performed between parameters.

Results

Characteristics of patients

Table 1 shows characteristics of patients involved in the study, including age, body mass index (BMI), and gender ratio. Data are shown as mean ± SEM.

Table 1. Patient characteristics.

Parameter	values
Age (Years)	39.4 ± 5.12 †
BMI (kg/m²)	29.6 ± 1.77 †
Male: female ratio	9: 6

BMI, body mass index. † Data expressed as mean ± SEM.

Serum uric acid in relation to allopurinol therapy

Allopurinol treatment was associated with a decline in serum UA, although non-significant (Fig. 1).

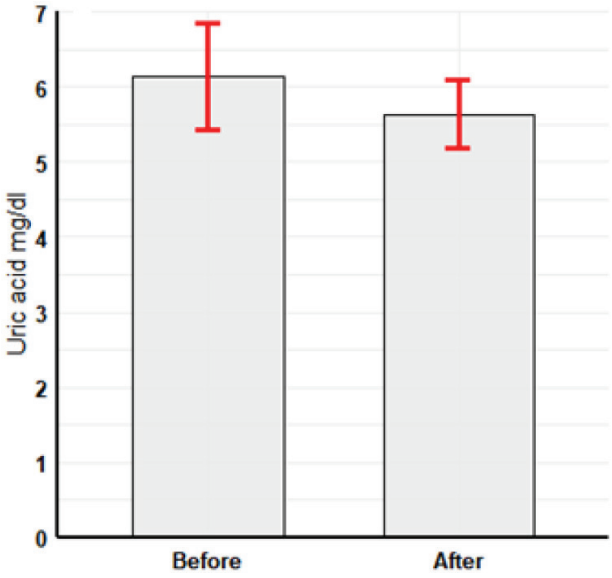

Figure 1. Effects of treatment with allopurinol on serum uric acid levels. A decline is demonstrated after therapy with allopurinol but is not statistically significant. Data are expressed as the mean ± SEM.

Serum haptoglobin, iron, ferritin and transferrin in relation to allopurinol

Although a decline in serum levels of Hp was registered in the present study after treatment with allopurinol, the change was not statistically significant (Fig. 2). Allopurinol use was associated with a significant rise in SI levels (P < 0.05) (Fig. 3). There were no significant differences in levels of FT after treatment compared to pre-treatment levels (Fig. 4). Similarly, use of allopurinol was not associated with any significant changes of

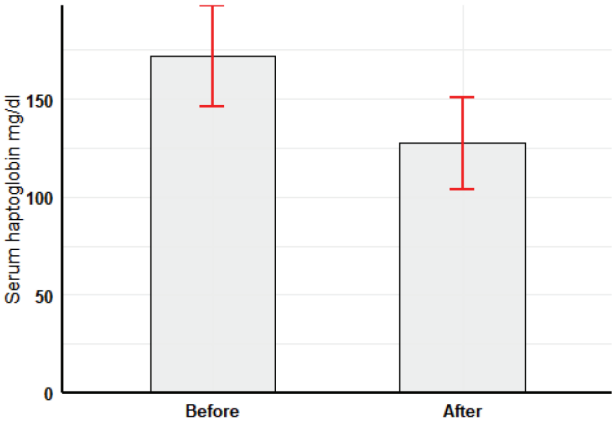

Figure 2. Effects of treatment with allopurinol on serum haptoglobin levels. A statistically non-significant decline is demonstrated after therapy with allopurinol. Data are expressed as the mean ± SEM.

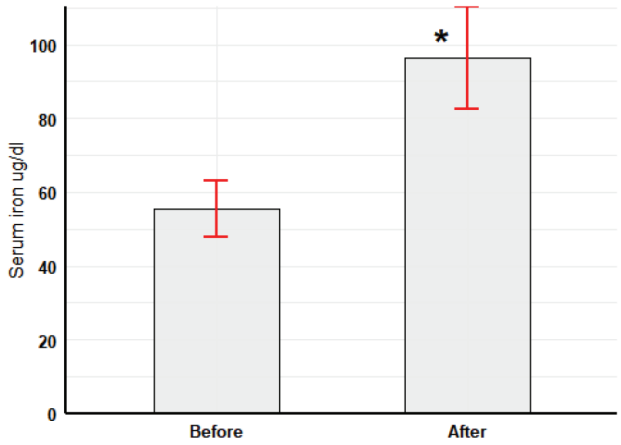

Figure 3. Effects of treatment with allopurinol on serum iron levels. Statistically significant rise (*$P < 0.05$) is demonstrated after therapy with allopurinol. Data are expressed as the mean ± SEM.

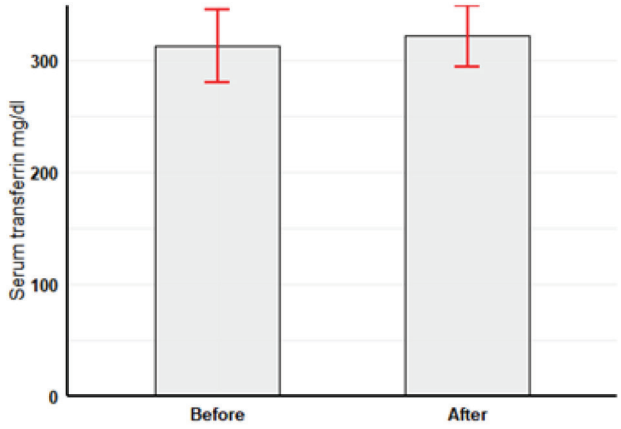

Figure 5. Effects of treatment with allopurinol on serum transferrin levels. A statistically non-significant change is demonstrated after allopurinol therapy. Data are expressed as the mean ± SEM.

serum TF post-treatment levels compared to baseline values (Fig. 5).

Correlations between parameters

Serum UA and iron did not show a significant correlation with any parameter in the study. Significant correlations between other parameters were also seen: Baseline values of Hp and FT were correlated positively (r = 0.65), additionally baseline values of TF and FT were negatively correlated (r = -0.78). Baseline values of Hp and TF were negatively correlated (r = -0.58). post-treatment levels of TF and FT were negatively correlated (r = -0.79).

Discussion

The major finding of the current study is that with suppression of XO activity via allopurinol in renal stone patients, UA showed an insignificant decline, while other iron-related parameters registered non-significant changes, save for serum iron.

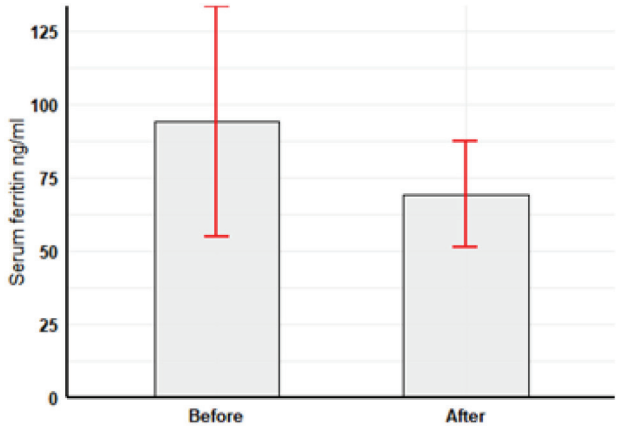

Figure 4. Effects of treatment with allopurinol on serum ferritin levels. A decline is demonstrated after allopurinol therapy but is not statistically significant. Data are expressed as the mean ± SEM.

The UA levels in human range between 3.5–7 mg /dl. High UA concentrations pose the risk of uric lithiasis in the urinary system (Wiederkehr and Moe 2011). While formation of renal stones do occur in HU patients, how HU and renal stone are interrelated is still unclear, other associated factors like low urine pH and hyperuricosuria significantly affect urolithiasis process (Tanaka et al. 2017). Allopurinol in the current study was indicated whether oxalate or urate stones were encountered. This has its logic in the way allopurinol functions. As an XOI, it blocks both urate generation in hyperuricemic or hyperuricosuric patients, and XO-catalyzed formation of oxalate from glycolate, hence reducing urinary levels of CaOx (Yasui et al. 2001). Accordingly, the drug is of benefit in lithiasis as well as in HU. Additionally, it is of utility in reducing cardiovascular and CKD risks, which provides firm grounds for using it urolithiasis patients especially when risk factors exist (Bove et al. 2017). It follows that allopurinol may be indicated for treatment even though overt HU is not detected.

In the present study, allopurinol administered to renal stone patients was associated with an insignificant decline in UA serum levels. Several factors may contribute to this finding. By definition, HU refers to when levels of serum UA exceed 7 mg/dl in men and 6 mg/dl in women (Desideri et al. 2014; Huang et al. 2017). XO suppression induced by allopurinol therapy may lead ultimately to reduced production and thus low serum level of urate (Chen et al. 2016); however, UA status is mainly controlled by both its rates of production and renal excretion, and while other factors may contribute to a rise in UA level, majority of HU cases are caused by diminished UA kidney excretion (Liu et al. 2021). In the current study, however, serum UA was non-significantly altered, and current study readings of UA approached the 6 mg/dl; thus as major contribution for maintaining UA level comes from renal excretion, rather than production, and unless renal function is overtly impaired, suppression of production (by the drug) should produce a minor effect, which we argue is the case in present study findings.

Furthermore, previous studies addressed lowering serum UA by means of allopurinol to a target value of < 6 mg/dl (which is the target outcome for gout) (Shoji et al. 2004; Halpern et al. 2009; Wu et al. 2009; Sundy et al. 2011); the drug was found inferior (to another XOI, febuxostat) in further reducing UA to below 5 mg/dl (Singh et al. 2015); hence, further reduction, especially in the related condition of nephrolithiasis, is not feasible by standard doses given, and since our patients were already approaching the limit of < 6 mg/dl achievable by the dose applied in the present study, non-significant changes in serum UA are expected and were actually noted.

Lack of significant hypouricemic effect in the present study may be thus dose-related. The 300 mg/day dose used in current study was shown in previous reports to be insufficient to a achieve a target level of UA of less than 6 mg/dl in more than half of treated patients (Watts 1966; Qu et al. 2017). In a large scale study, Jennings et al. (2014) found that one-third of treated patients required upscaling of dose to reduce UA level to below 6 mg/dl target. In other previous reports, patients were started on a lower dose (100 mg) to be up-titrated later to (300 mg/day) but up to half patients failed to show a decline in UA to less than 6 mg/dl (Becker et al. 2005; Jordan et al. 2007). Additionally, allopurinol failed to reduce UA to less than target 6 mg/dl in up 60–80% of patients in other reports (Suresh and Das 2012; Gustafsson and Unwin 2013), which highlighted the need for further therapy with other agents. This is in line with present study of minimal hypouricemic effect allopurinol exerted on our urolithiasis patients, especially taking into consideration the baseline values of UA which were not sufficiently elevated to affect magnitude of response at the used dose.

Renal insufficiency is common in HU-related conditions which can limit the hypouricemic effect of XOIs like allopurinol (Faisal et al. 2020) since least a fraction of urolithiasis patients are in fact renal disease patients. As such, serum UA elevation reflects and is a marker of, suboptimal renal excretion (Skoczyńska et al. 2020). Present study ranges were not below or beyond normal limits by a significant degree; and renal function, as judged by degree of derangement of UA levels, was probably not compromised. This may account for nonsignificant changes seen in this parameter after allopurinol treatment, as otherwise elevated UA levels should be noted at baseline. However, it is notable that HU do not demonstrate a constant association with renal stones despite being a major urolithiasis risk factor (Heilberg 2016). The urate urolithiasis and low pH of urine are in fact underlying renal signs of insulin resistance (IR) (Heilberg 2016); thus XO inhibition could contribute to only slight changes in urate status in this context.

Evidence suggests that iron metabolism is a major etiological contributor to HU development (Su et al. 2016). On the other hand, preexisting infectious or inflammatory conditions may affect iron status interpretation as acute phase response has an impact on most iron status biomarkers (Suchdev et al. 2017). Monosodium urate crystals, precipitated by elevated serum UA, trigger inflammation in affected areas (Yang et al. 2016), among iron status parameters, FT was shown to rise during infection and chronic inflammation (Feelders et al. 1998; Yang et al. 2016). Particularly in renal stone patients, it was suggested that local injury intra-renally mediated by nephrolithiasis, initiates a local then a systemic inflammatory state which in turn influences iron status (Wang et al. 2001); under such conditions, accurately interpreting such indices could be elusive.

Iron status in urolithiasis patients is an area not studied extensively. Trace elements contribution to renal stone formation is still unknown with certainty. Limited number of studies addressed relation between iron and renal stones, some reports indicated no effect of iron on CaOx stones (Meyer and Angino 1977), while others reported renal iron excretion in oxalate stone patients to be superior to healthy controls (Elliot and Ribeiro 1973). In the study by Atakan et al. (2007), SI level was higher in healthy controls in contrast to renal stone patients, and authors suggested that iron did not inhibit but rather promoted the generation of CaOx stones (Atakan et al. 2007). This indicates controversy in previous reports.

In the present study, SI was significantly changed in association with XO inhibition. This is consistent with reports showing that use of allopurinol elevated SI in gouty patients (Powell and Emmerson 1966).In contrast, SI levels in those maintained on allopurinol therapy were not reported to go beyond normal limits even for therapy lasting months to years, in other studies (Emmerson 1966; Rundles et al. 1966; Greene et al. 1969). Other than SI, the current study demonstrated an overall minimal impact of allopurinol on iron metabolism. No measurable effect of this drug on aspects of iron metabolism, absorption, or storage were reported in patients with HU or gout (Boyett et al. 1968). Any deleterious (or otherwise) effects of allopurinol on iron metabolism were not reported (Emmerson 1966). Iron hepatic storage in rats fed allopurinol was found to be higher compared to controls in another experiment (Powell 1966) indicating XO inhibition may play a role in the process. All these findings are largely in line with present study findings. However, no relevant more recent works specifically on renal stone patients could be located for comparison with the current study.

Exposure to iron was shown to enhance XO activity, which was postulated to explain a causal relationship of iron with UA seen in some reports (Fatima et al. 2018). Alenezi et al. (2020) suggested that elevated UA directly correlates with a rise in levels of SI, additionally, FT itself was found to be related to UA in several large-scale studies (Ghio et al. 2005; Li et al. 2018), even to the point that an increase in UA was suggested to be a biomarker of iron overload (Mainous et al. 2011). Direct link between acute iron exposure and UA level was reported also in thalassemia major patients (Livrea et al. 1996). All this evidence suggests inconsistency with current study findings, but

this may be due to that these reports were carried out on different categories of patients, and that XO inhibition is not a reported intervention used in these studies. In support of this view, some authors reported that reciprocal relationship is more logical between urate which chelates iron on one hand, and iron, on the other, which exerts modulating effect on the activity of XO and hence controls to some extent production of UA (Ghio et al. 2002). Body iron condition may actually control urate status rather than otherwise, and suppression of XO by ULT may not produce evident changes if iron status is disrupted. This in general is explanatory of and consistent with, present study findings.

Increased serum levels of iron would increase the level of saturated transferrin, this in turn increases XO activity, (Martelin et al. 2002), this in turn will lead to increased serum UA (Zeng et al. 2008). Iron deficiency will produce the opposite effect (Ghio et al. 2002; Kelley and Amy 1984), hence decreased UA will manifest. Ghio et al. (2002) reported that in their study iron appeared to control lung OX activity such that high iron levels increased the enzyme activity and thus levels of UA and vice versa. Conversely, XO is known to catalyze the release of ferritin iron and chelation to ferrous form, this iron-mobilizing capacity of XO indicates that XO activity increase concentration of SI and urate and thus its inhibition has an opposite effect (Bolann and Ulvik 1987), this is inconsistent with (insignificant) urate decline in the present study, but it probably reflects a superior effect of allopurinol than the drive by SI on its levels.

In the current study no significant correlation was found between UA, SI and FT, This is inconsistent with previous reports like Fatima et al.(2018) and Wang et al. (2020); however, inconclusive evidence is reported in other populations (Budzyń et al. 2011; Dasgupta et al. 2013); given the population heterogeneity in these studies, such correlations may depend on the disease population under study, especially as these populations involved non-lithiasis patients and XO inhibition was not an intervention in the studies.

Haptoglobin (Hp), a marker utilized in hemolytic conditions, was measured in the present study. The XO activity is a major source of reactive and oxidative species that contribute to hemolytic diseases (Schmidt et al. 2019), thus its suppression is a logical step in the course of maintaining blood cell integrity in susceptible populations. Polymorphism of Hp affects iron metabolism (Langlois et al. 2000; Beutler et al. 2002; Kasvosve et al. 2002; Van Vlierberghe et al. 2004), and different Hp phenotypes correlated with higher SI, transferrin saturation and FT concentrations more than others (Na et al. 2006). Based on this and on its biological function, Hp obviously affects iron turnover and hence iron metabolism and status. On the other hand, Hp, being an acute phase protein, is inducible by IL-6 during inflammatory states (Marro et al. 2007), in a manner similar to ferritin. No significant correlation or changes in Hp after ULT in the present study were observed. Previous reports addressed this issue with a phenotype-centered approach and some were consistent with current study. Carter et al.(2003) examined the influence of Hp polymorphism on, and correlation with, iron metabolism, which they found not to influence iron status. Also in line with current study findings, Hp and iron status markers were not correlated significantly in previous reports (Beutler et al. 2002; Kasvosve et al. 2002). XO contributes to hemolytic diseases, plasma activity of this enzyme correlated with oxidative stress (OS) markers and in an experiment on a rodent sepsis model, where allopurinol and desferrioxamine both blunted the increase from free radicals generated after hemolysis (Chatterjee et al. 2011). However, in the context of current work no deleterious effect was seen in terms of markers measured.

It should be mentioned that a significant part of XO enzyme is transferred to and is bound by endothelial surfaces, such trapped XO enzyme is resistant to the action of allopurinol, and high drug concentrations, much in excess of those achieved by therapeutic doses are needed to achieve half the inhibition obtained when allopurinol acts on free XO, in comparison (Kelley et al. 2004). Thus, both the doses given need to be up-titrated and baseline values of parameters need to be evidently abnormal for changes to be seen; even at this point, given the aforementioned notes, changes observed may be minimal especially for urolithiasis patients who are otherwise healthy. Despite its relatively minimal influence on iron metabolism in settings similar to current study's setting, a case still exists for allopurinol use, as the latter by virtue of XO inhibition, may have benefits extending beyond controlling urate level such as lowering serum creatinine or blood pressure (Benn et al. 2018), and improving endothelial function in at-risk populations (Yiginer et al. 2008).

The current study has some limitations. The sample size is relatively small, and there was no control group for comparison. Beneficial or deleterious effects are probably dose-related, stratification according to dose as well as other factors like age and gender could be of utility, however, sample size considerations dictated otherwise. As inflammation can preclude some of the parameters involved in the current study from being interpreted adequately, robust markers for inflammation should have been measured or its effects accounted for. Interpretation of study findings should take into account its limitations to draw sound and accurate conclusions, and future studies should address current work limitations.

Conclusions

In conclusion, when administered for urolithiasis patients, allopurinol was associated with minimal effect with regard to serum levels of uric acid, ferritin, transferrin and haptoglobin, after 6-month therapy, while serum iron was significantly changed. No correlation between levels of uric acid and iron with other parameters was found. As

discussed, lack of significant changes can be attributed to several factors. Despite this, and as XO inhibition is linked to other beneficial merits independent of drug effects on urate, and in light of minimal effect on iron metabolism, save for iron, a case still exists for using the drug in different populations regardless of iron status. However, when benefit is linked to reduction in uric acid, patients with renal stones, especially those approaching normouricemia, are probably minimally benefiting from the drug. To elaborate on present study results and to explore the underlying mechanisms, larger-scale prospective studies, possibly with a second study arm for another XO inhibitor (febuxostat) may be warranted.

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