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Nitisinone arrests ochronosis and decreases rate of progression of Alkaptonuria: Evaluation of the effect of nitisinone in the United Kingdom National Alkaptonuria Centre.

Title: Nitisinone arrests ochronosis and decreases rate of progression of Alkaptonuria: evaluation of the effect of nitisinone in the United Kingdom National Alkaptonuria Centre

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Abstract

Question: Does Nitisinone prevent the clinical progression of the Alkaptonuria?

Findings: In this observational study on 39 patients, 2mg of daily nitisinone inhibited ochronosis and significantly slowed the progression of AKU over a three-year period.

Meaning: Nitisinone is a beneficial therapy in Alkaptonuria

Background: Nitisinone decreases homogentisic acid (HGA), but has not been shown to modify progression of Alkaptonuria (AKU).

Methods: Thirty-nine AKU patients attended the National AKU Centre (NAC) in Liverpool for assessments and treatment. Nitisinone was commenced at V1 or baseline. Thirty nine, 34 and 22 AKU patients completed 1, 2 and 3 years of monitoring respectively (V2, V3 and V4) in the VAR group. Seventeen patients also attended a pre-baseline visit (V0) in the VAR group. Within the 39 patients, a subgroup of the same ten patients attended V0, V1, V2, V3 and V4 visits constituting the SAME Group. Severity of AKU was assessed by calculation of the AKU Severity Score Index (AKUSSI) allowing comparison between the pre-nitisinone and the nitisinone treatment phases.

Results: The ALL (sum of clinical, joint and spine AKUSSI features) AKUSSI rate of change of scores/patient/month, in the SAME group, was significantly lower at two (0.32±0.19) and three (0.15±0.13) years post-nitisinone when compared to pre-nitisinone (0.65±0.15) ($p<0.01$ for both comparisons). Similarly, the ALL AKUSSI rate of change of scores/patient/month, in the VAR group, was significantly lower at one (0.16±0.08) and three (0.19±0.06) years post-nitisinone when compared to pre-nitisinone (0.59±0.13) ($p<0.01$ for both comparisons). Combined ear and ocular ochronosis rate of change of scores/patient/month was significantly lower at one, two and three year's post-nitisinone in both VAR and SAME groups compared with pre-nitisinone ($p<0.05$).

Conclusion: This is the first indication that a 2mg dose of nitisinone slows down the clinical progression of AKU. Combined ocular and ear ochronosis progression was arrested by nitisinone.

Introduction

Alkaptonuria (AKU) is a rare genetic deficiency of homogentisate dioxygenase (HGD), characterised by high circulating homogentisic acid (HGA), some of which is deposited in connective tissue as a pigmented polymer, during a process termed ochronosis [1,2]. The effects of ochronosis include premature arthritis, lithiasis, cardiac valve disease, fractures, muscle and tendon ruptures and osteopenia [3,4].

Current therapy is only palliative [5]. A potential agent called nitisinone has been shown to decrease circulating HGA [6,7,8] and to inhibit ochronosis in AKU mice [9,10]. Nitisinone which inhibits p-hydroxyphenylpyruvate dioxygenase, the enzyme leading to formation of HGA, has been used for more than twenty years in the treatment of type-1 hereditary tyrosinaemia (HT-1) [11,12]. The dose of nitisinone that decreases HGA by greater than 95% is 2 mg daily, based on the experience of using nitisinone in the National Institute of Health, USA [6,7,8], and the SONIA-1 clinical study [13], and approximately ten to fifty times lower than the doses used in HT-1. NHS England has approved the use of off-label nitisinone 2 mg daily for the management of AKU in the National Alkaptonuria Centre (NAC) Liverpool, UK [14].

A previous study employing 2 mg dose of nitisinone, failed to demonstrate a benefit on a single non-metabolic outcome, hip rotation [8]. The NAC is collecting data from a large number of assessments to calculate the severity of AKU using a validated semi-quantitative composite score termed AKUSSI (alkaptonuria severity scoring index) [15,16], which increases the likelihood of detecting an effect of nitisinone.

Subjects and Methods

The NAC was established in the Royal Liverpool University Hospital and funded by the Highly Specialised Services, NHS England, in April 2012. The lead author's institutional audit committee approved the analysis of the NAC data (Audit no. ACO3836).

Thirty-nine AKU patients attended the National AKU Centre (NAC) in Liverpool (Figure 1). Varying numbers attended yearly visits in this VAR group (or VAR; variable number of patients at each visit). Nitisinone 2mg was commenced at baseline (V1). Systematic assessments were carried out at all visits. Thirty-nine, 34 and 22 AKU patients completed 1, 2 and 3 years of monitoring respectively (V2, V3 and V4) after starting nitisinone. Seventeen patients (7 female and 10 male) also attended a pre-baseline visit (V0) in the VAR group; the duration between the V0 and V1 was 32.2 (±2.3) months. V1 (n = 39; mean age 47.3 (±2.3) years; 15 females; 24 male), V2 (n = 39; mean age 48.3 (±2.3) years; 15 females; 24 male), V3 (n = 34; mean age 48.7 (±2.6) years; 14 females; 20 male), and V4 (n = 22; mean age 47.3 (±3.4) years; 9 females; 13 male) visits in the VAR group had a full data set in which the AKUSSI was calculated.

Within the 39 patients, a subgroup of the same ten patients attended V0, V1, V2, V3 and V4 visits constituting the SAME Group; the duration between V0 and the V1 was 36.7±2.2 months. Attendance thereafter in the NAC was once a year.

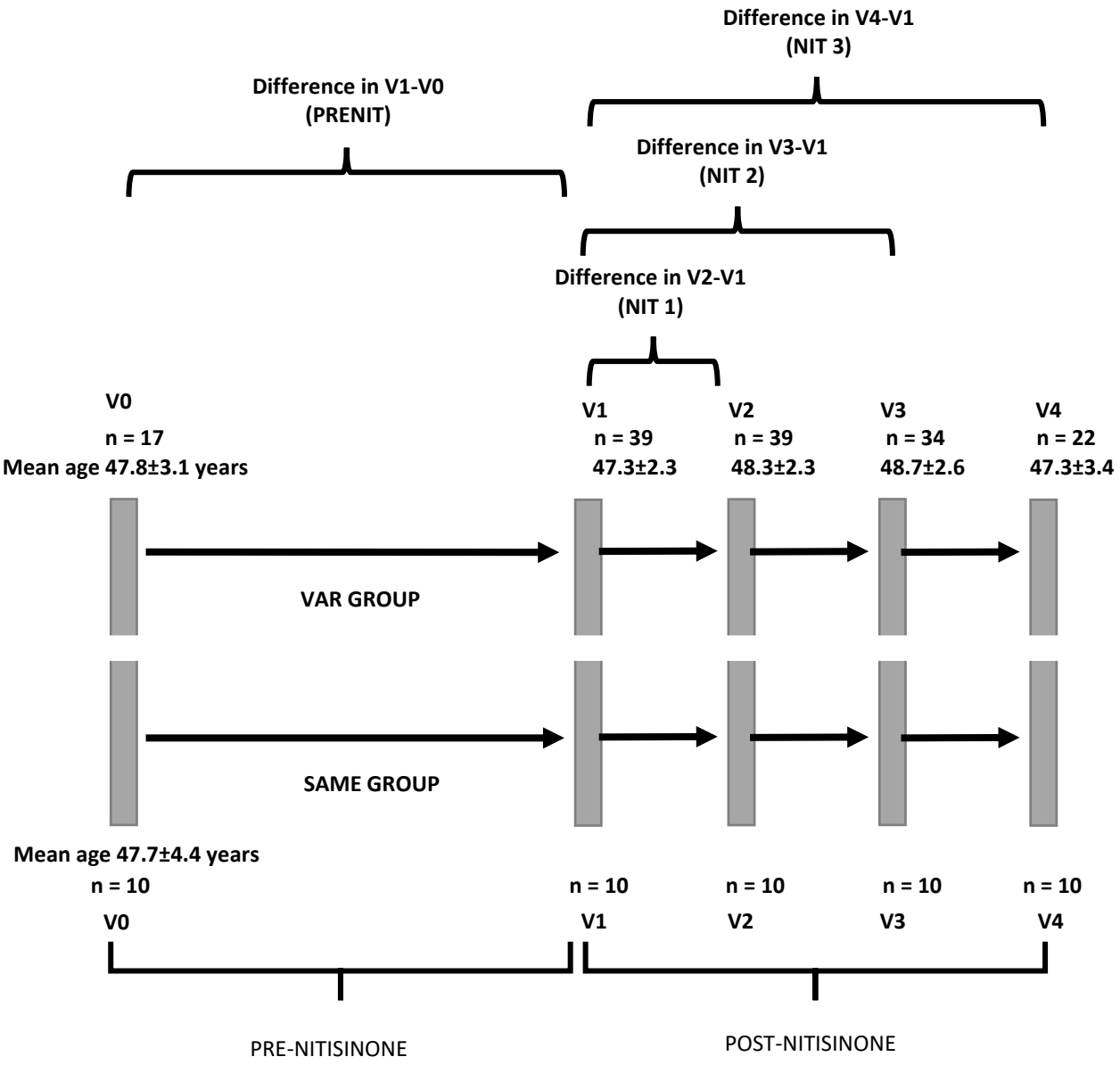

Figure 1. Plan of the National Alkaptonuria Service: *The VAR group V0 visit consisted of the 10 patients from the SAME group plus seven additional patients who attended the NAC twice without receiving nitisinone. The SAME refers to ten patients attending the research study between 2008 and 2011. The V1, V2, V3 and V4 refer to yearly visits to the NAC. The NIT 1, NIT 2 and NIT 3 refer to change scores per patient per year after one, two and three years of nitisinone therapy. The numbers of patients in each group, their mean age and years of follow-up are also shown in the figure.

Change in scores between V0 and V1, V1 and V2, V1 and V3, and V1 and V4 represent follow-up without nitisinone, as well as one, two and three years of nitisinone therapy, termed PRENIT, NIT 1, NIT 2, and NIT 3 respectively.

Assessments used in AKUSSI and assigned scores [13,14] (Table 1): Assessments and investigations detailed in Table 1 were carried out at V0, V1, V2, V3 and V4. The AKUSSI was developed during a study of AKU (UK Research Ethics Committee Number 07/Q1002/111) [15,16]. Ear and eye ochronosis, calculi (renal, prostate), osteopenia, fracture, ruptures (tendon/ligament/muscle), aortic valve disease, hearing impairment collectively constitute the CLIN category. Pain and scintigraphy scores in 14 joint areas, arthroscopy and joint replacements, comprise the JOINT category. Pain and scintigraphy scores in 6 areas including the spine, pubis and ribs comprise the SPINE category. The sum of CLIN, JOINT and SPINE scores constitute the ALL category. There is no upper maximal score as some features such as fractures are not finite. The original AKUSSI was modified by having improved scoring of eye ochronosis (superficial conjunctival and deep scleral), each episode of renal stone, aortic valves disease (mild, moderate and severe stenosis as well as sclerosis), a T-score equal to or less than -1.1 for osteopenia, score for kyphosis given if Cobb angle was greater than 30º, and score for scoliosis given if Cobb angle was greater than 10º; in addition, some features such as heart failure, Parkinson's disease, atrial fibrillation, other cardiac arrhythmias, strokes, skin pigment, teeth pigment, middle ear pigment, laryngeal pigment, and salivary stone, were excluded to improve consistency.

FEATURE	TEST	FEATURE	TEST
<u>Eye ochronosis:</u>			
Right eye nasal*	PHOTO	Left eye nasal*	PHOTO
Right eye temporal*	PHOTO	Left eye temporal*	PHOTO
<u>Ear ochronosis:</u>			
Right ear**	PHOTO	Left ear**	PHOTO
Prostate stones (4 per episode)	US/ HISTORY	Kidney stones (4 per episode)	US/ HISTORY
Osteopenia (4)	CT-BMD	Hearing impairment (4)	HISTORY
Aortic sclerosis (6), Aortic stenosis (mild, moderate, severe) (8, 10, 12)			ECHO

Fracture (8 per fracture)	HISTORY	Muscle rupture (8 per rupture)	HISTORY
Ligament rupture (8 per rupture)	HISTORY	Tendon rupture (8 per rupture)	HISTORY
CLINICAL AKUSSI			
JOINT PAIN score (1 for each large joint area; 14 large joint areas)			HISTORY
Scintigraphic scan joint score (2 for each large joint; 14 large joint areas)			18F-PETCT
Number of arthroscopies (2 each)			HISTORY
Number of joint replacements (4 each)			HISTORY
JOINT AKUSSI			
SPINAL PAIN score (2 each for cervical, thoracic, lumbar, sacroiliac)			HISTORY
Scintigraphic scan spine score (6 areas; 4 points for each area; pubic symphysis, costochondral, lumbar, thoracic, cervical, sacroiliac)			18F-PETCT
Kyphosis (4)			X-RAY
Scoliosis (4)			X-RAY
SPINE AKUSSI			
ALL AKUSSI (CLINICAL+JOINT+SPINE)			

Table 1. AKUSSI scoring in the NAC. The various clinical features were scored in the manner indicated in the table. The assessments included subjective pain scoring, photographs, history, ultrasound abdomen, echocardiogram, dual energy x-ray absorptiometry, x-ray spine and ^{18F}PETCT scan. *Eye pigmentation: 1, 2 and 3 points for slight, moderate and marked conjunctival pigmentation and 4, 6 and 8 points for scleral pigmentation; **Ear pigmentation: 2 and 4 points for slight and marked pigmentation.

Off-label nitisinone usage: Nitisinone was commenced on day 3 of V1. Fasting serum and 24-hr urine samples were collected on days 2 and 4 and patients discharged on nitisinone 2 mg alternate days. Fasting serum and 24-hr urine were collected at month 3 post-nitisinone before patients started nitisinone 2 mg daily and at month 6 post-nitisinone. Fasting serum and 24-hr urine were thereafter collected at V2, V3, and V4. Supportive therapies such as analgesia (including neuromuscular blocks) and lifestyle advice were also provided in the NAC. Therefore, AKUSSI scores for JOINT, SPINE and ALL were also calculated without the pain scores (JOINTNP: NP meaning no pain scores, SPINENP and ALLNP) to determine if these treatments affected the results. Physiotherapy reinforced appropriate exercises to support optimal function. Dietetic management ensuring optimal protein intake was employed to minimise tyrosinaemia post-nitisinone.

Chemical analysis: Only samples from 2012 onwards were available for assay. HGA was measured on acidified 24-hr urine (u-HGA_{24}) and acidified serum (s-HGA) samples from each visit as previously described by tandem mass spectrometry [17,18]**.**

Statistical analysis:

Multiple visit data were subjected to repeated measures of variance ANOVA, and unpaired data by ANOVA with post hoc correction for multiple comparison as appropriate (Tukey Kramer).

Results

All patients had increased urine HGA confirming the diagnosis of AKU at V1. u-HGA_{24} concentrations decreased by 80.2 to 93.9% at all follow-up visits compared to V1 for the SAME and the VAR groups ($p<0.01$) (Figure S1). Similarly, s-HGA decreased by 80.2-89.3% at follow-up visits as compared to V1 respectively in both the SAME and VAR groups ($p<0.01$; Figure S2).

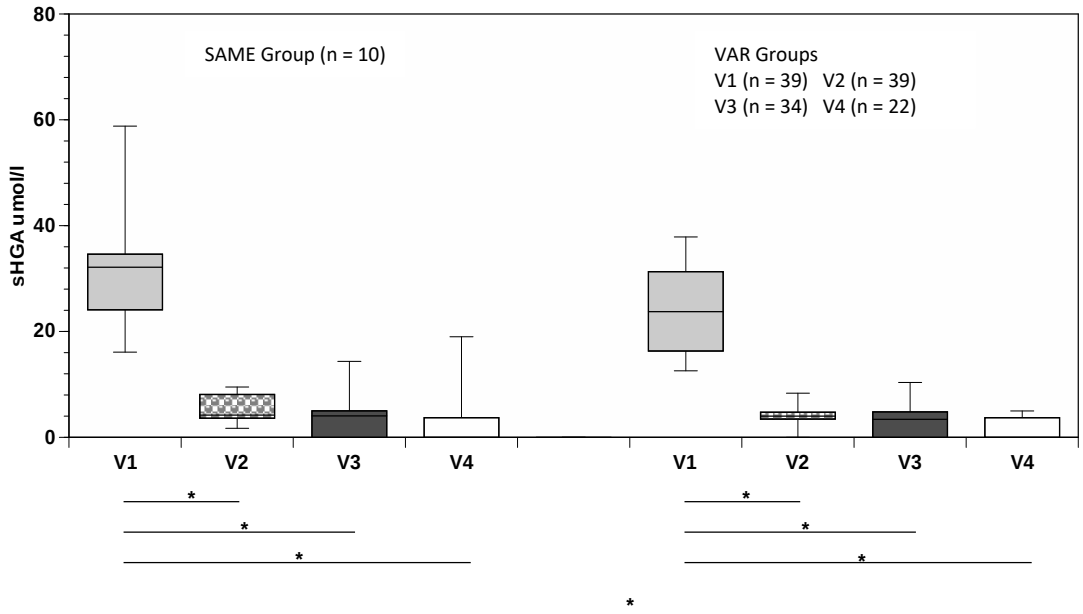

Figure S1. s-HGA concentrations in SAME and VAR groups, Pre- and Post-Nitisinone. Scores are shown as box plots with and interquartile range. The level of significance of results is shown as *$p<0.001$.

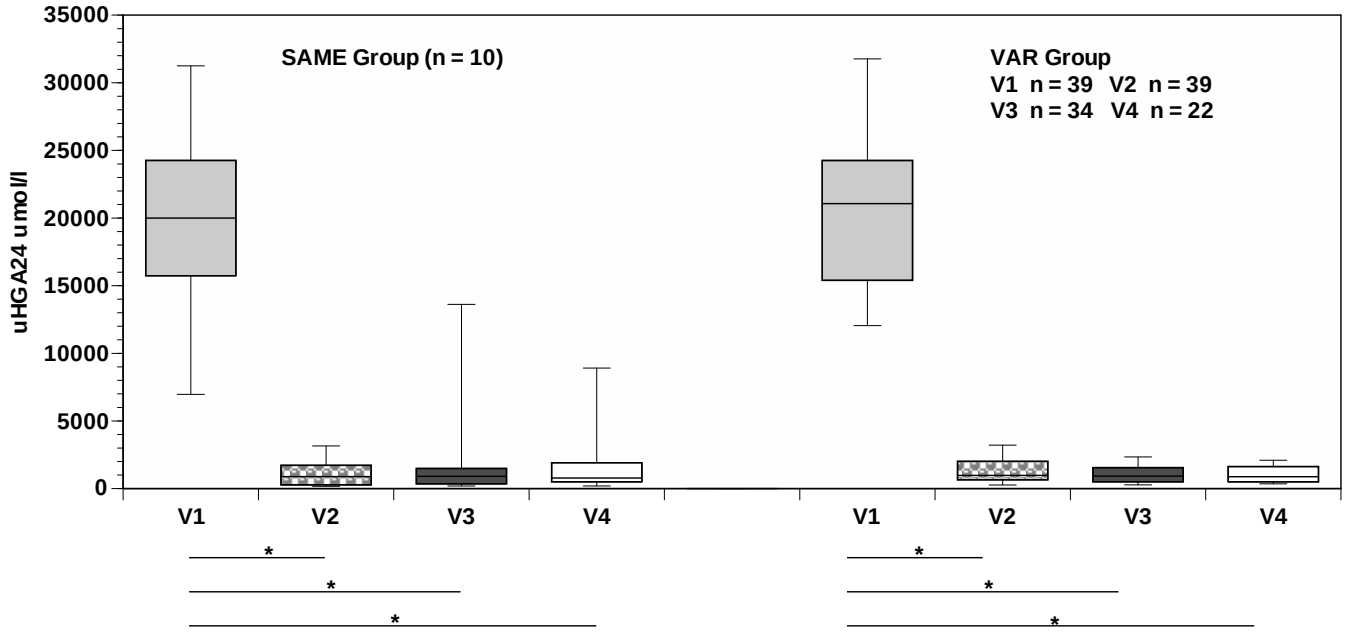

Figure S2. u-HGA_{24} concentrations in SAME and VAR groups, Pre- and Post-Nitisinone. Scores are shown as boxplots with interquartile range. The level of significance of results is shown as *p<0.001.

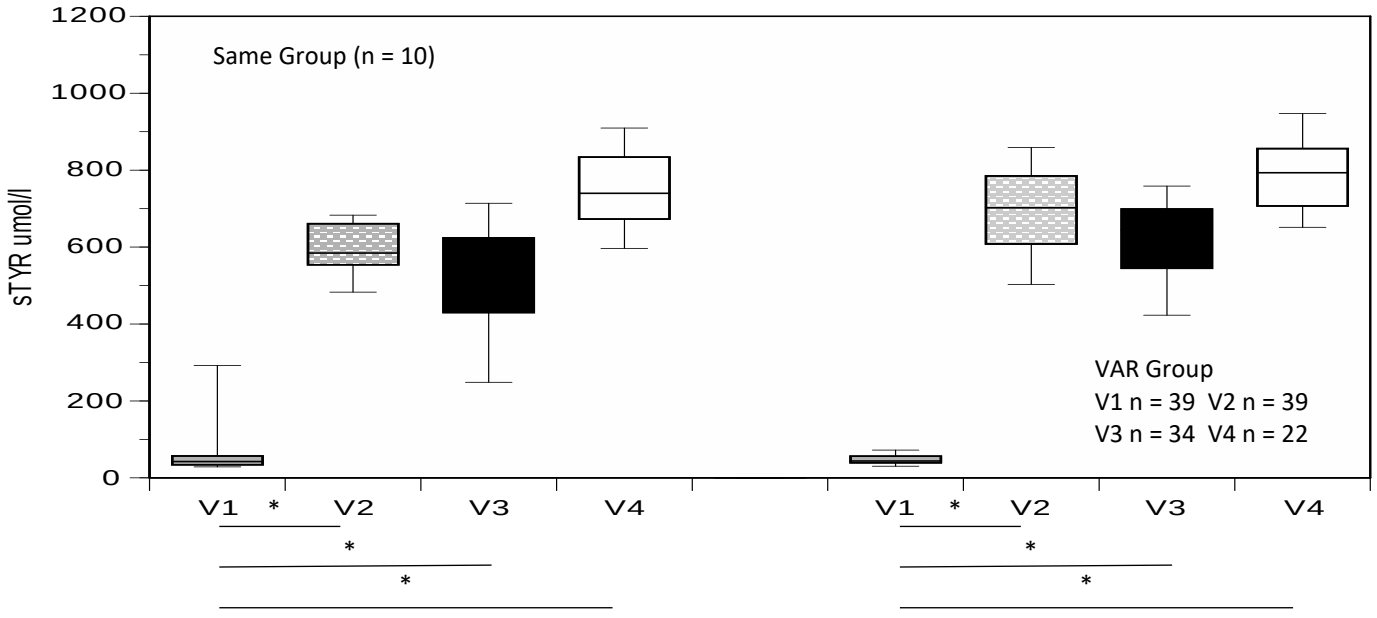

Figure S3. s-TYR concentrations in SAME and VAR groups, Pre- and Post-Nitisinone. Scores are shown as boxplots with interquartile range. The level of significance of results is shown as *p<0.001.

Table S1. Numbers of events in Clinical AKUSSI category without pigment change in the SAME and VAR groups. Please note varying event numbers in varying numbers of patients in the VAR group.

	SAME GROUP					VAR GROUP				
	V0 (n = 10)	V1 (n = 10)	V2 (n = 10)	V3 (n = 10)	V4 (n = 10)	V0 (n = 17)	V1 (n = 39)	V2 (n = 39)	V3 (n = 34)	V4 (n = 22)
Prostate Stone	1	1	1	1	1	5	13	13	11	5
Renal stone	2	2	2	2	2	6	6	6	7	6
Osteopenia	7	10	10	10	9	11	27	27	27	14
Fracture	7	9	10	11	14	9	21	24	23	21
Tendon rupture	0	0	0	0	0	1	5	7	6	0
Muscle rupture	3	3	3	3	3	5	5	7	8	5
Ligament rupture	4	5	5	5	5	6	8	8	8	6
Aortic sclerosis	3	5	4	4	4	6	14	12	10	8
Mild Aortic Stenosis	1	2	3	2	2	2	3	5	6	3
Moderate Aortic Stenosis	0	0	0	0	1	1	1	0	0	1
Severe Aortic Stenosis	0	1	1	1	1	0	2	2	1	1
Hearing impairment	3	4	4	5	5	4	11	11	12	10
Arthroplasty	6	12	15	17	17	12	36	39	43	33

SAME Group (Table 2): At baseline the following numbers of events (in brackets) were observed in the SAME group: prostate stone (1), renal stones (2), osteopenia (10), fractures (9), tendon rupture (0), muscle rupture (3), ligament rupture (5), aortic sclerosis (5), aortic stenosis (3), hearing loss (4) and joint replacements (12) (**Table S1**). There was a significant increase in JOINT, JOINT WITHOUT PAIN, SPINE, ALL and ALL WITHOUT PAIN categories between V0 and V1 (Table 2). The CLIN and

SPINE WITHOUT PAIN showed a similar trend in the V0 and V1 comparisons. None of the other comparisons was significant.

SAME PATIENT NUMBER GROUP (SAME) (n = 10) [Mean(SEM)]					
Categories	V1	V0	V2	V3	V4
CLIN	40.3 (7.7)	31.6 (6.3)	40.2 (8.3)	40.4 (8.7)	42.7 (10.7)
JOINT	25.9 (3.4)	19.1 (3.0)*	28.5 (2.8)	28.1 (2.7)	27.5 (3.5)
JOINT WITHOUT PAIN	20.0 (2.5)	14.0 (2.2)*	22.6 (2.5)	22.8 (2.6)	22.2 (2.9)
SPINE	26.0 (2.8)	19.2 (2.9)*	27.2 (3.2)	28.2 (3.3)	27.4 (3.3)
SPINE WITHOUT PAIN	20.8 (2.1)	16.0 (2.6)	22.0 (2.5)	22.4 (2.4)	22.0 (2.7)
ALL	92.2 (12.6)	69.8 (11.0)**	95.9 (12.8)	96.7 (13.3)	97.6 (15.8)
ALL WITHOUT PAIN	82.1 (11.0)	61.5 (10.1)**	84.8 (11.5)	85.6 (12.2)	86.9 (14.7)
VARIABLE PATIENT NUMBER GROUP (VAR) [Mean(SEM)] (mean and SEM for any V category are different in each column)					
Categories	V1 vs V0 (n=17)	V1 vs V2 (n=39)	V1 vs V3 (n=34)	V1 vs V4 (n=22)	
CLIN	39.7(5.9) vs 32.5 (5.3)	29.5 (3.3) vs 29.6 (3.5)	29.9 (3.5) vs 31.2 (3.8)	28.9 (4.5) vs 31.1 (5.5)	
JOINT	26.8(3.2) vs 20.6 (3.1)	22.0 (1.9) vs 22.6 (1.9)	21.4 (2.1) vs 25.1 (1.9)	22.5 (2.9) vs 24.3 (3.2)	
JOINT WITHOUT PAIN	20.8(2.7) vs 16.0 (2.6)	16.5 (1.4) vs 17.3 (1.5)	16.5 (1.6) vs 19.8 (1.5)	17.4 (2.2) vs 19.2 (2.6)	
SPINE	26.0(2.5) vs 20.2 (2.7)	24.6 (1.8) vs 24.7 (1.9)	24.4 (2.0) vs 26.2 (2.1)	22.9 (2.7) vs 24.5 (3.0)	
SPINE WITHOUT PAIN	20.5(1.9) vs 16.7 (2.2)	20.1 (1.4) vs 20.4 (1.5)	20.0 (1.6) vs 21.3 (1.7)	18.7 (2.2) vs 19.8 (2.4)	
ALL	92.5(10.3)vs73.5(9.8)*	76.1 (6.0) vs 77.0 (6.1)	75.6 (6.5) vs 82.5 (6.6)	74.3 (8.9) vs 79.9 (10.1)	
ALL WITHOUT PAIN	80.2 (9.0) vs 64.5 (8.7)	66.0 (5.2)vs 67.3 (5.5)	66.4 (5.7)vs 72.3 (5.9)	65.0 (7.7) vs 70.1 (9.1)	

Table 2. SAME and VAR groups showing the scores [mean (SEM)] for the various components of the AKUSSI (all comparisons against V1, repeated measures ANOVA; n=10; Tukey Kramer multiple comparison), *p<0.05; ** p<0.01; *** p<0.001, **** p<0.0001. Mean and SEM for any VAR category are different in each column.

SAME Group score change over time (Table 3, Figure 2a): The score change per patient per month showed lower values for NIT-1, NIT-2 and NIT-3 compared against PRE-NIT in CLIN, ALL and ALL WITHOUT PAIN categories. These comparisons showed a similar lower trend from PRE-NIT to NIT-3 for all other categories. Change in combined ear and eye pigment scores was lower during nitisinone usage (Figure 3).

Table 3. SAME and VAR groups showing the change scores per patient per month (mean±SEM) for the various components of the AKUSSI (repeated measures ANOVA; n=10; Tukey Kramer multiple comparison), *p<0.05; ** p<0.01; *** p<0.001.

SAME PATIENT NUMBER GROUP (SAME) (n = 10)			
Categories	PRENIT vs NIT 1	PRENIT vs NIT 2	PRENIT vs NIT 3
CLIN	0.249 (0.08) vs 0.002 (0.08)***	0.249 (0.08)vs 0.004 (0.06)***	0.249 (0.08) vs 0.067 (0.1)**
JOINT	0.206 (0.08) vs 0.192 (0.12)	0.206(0.08) vs 0.088 (0.05)	0.206 (0.08) vs 0.041 (0.03)
JOINT WITHOUT PAIN	0.180 (0.07) vs 0.208 (0.09)	0.180 (0.07) vs 0.115 (0.06)	0.180 (0.07) vs 0.06 (0.03)
SPINE	0.192 (0.06) vs 0.104 (0.17)	0.192 (0.06) vs 0.09 (0.08)	0.192 (0.06) vs 0.039 (0.05)
SPINE WITHOUT PAIN	0.134 (0.04) vs 0.100 (0.15)	0.134 (0.04) vs 0.067 (0.07)	0.134 (0.04) vs 0.033 (0.05)
ALL	0.647 (0.14) vs 0.294 (0.17)*	0.647 (0.14) vs 0.184 (0.10)**	0.647(0.14) vs 0.146 (0.13)**
ALL WITHOUT PAIN	0.561 (0.11) vs 0.306 (0.15) *	0.561 (0.11) vs 0.186 (0.11)**	0.561(0.11) vs 0.159(0.14)***
VARIABLE PATIENT NUMBER GROUP (VAR)			
Categories	PRENIT vs NIT 1 n=17 vs 39	PRENIT vs NIT 2 n=17 vs 34	PRENIT vs NIT 3 n=17 vs 22
CLIN	0.221 (0.06)vs 0.016 (0.05)	0.221 (0.06) vs 0.053 (0.04)	0.221 (0.06) vs 0.061 (0.05)
JOINT	0.188 (0.06) vs 0.045 (0.06)	00.188 (0.06) vs 0.156 (0.04)	0.188 (0.06) vs 0.051 (0.03)
JOINT WITHOUT PAIN	0.159 (0.05) vs 0.066 (0.04)	0.159 (0.05) vs 0.135 (0.03)	0.159 (0.05) vs 0.053 (0.03)
SPINE	0.183 (0.05) vs 0.013 (0.06)	0.183 (0.05) vs 0.078 (0.03)	0.183 (0.05) vs 0.043 (0.03)
SPINE WITHOUT PAIN	0.273 (0.06) vs 0.026 (0.04)	0.273 (0.06) vs 0.054 (0.03)	0.273 (0.06) vs 0.031 (0.03)
ALL	0.591 (0.13) vs 0.074 (0.1)**	0.591 (0.13) vs 0.287 (0.06)	0.591 (0.13) vs 0.154 (0.07)*
ALL WITHOUT PAIN	0.485 (0.11) vs 0.108 (0.08)*	0.485 (0.11) vs 0.243 (0.06)	0.485 (0.11) vs 0.141 (0.08)

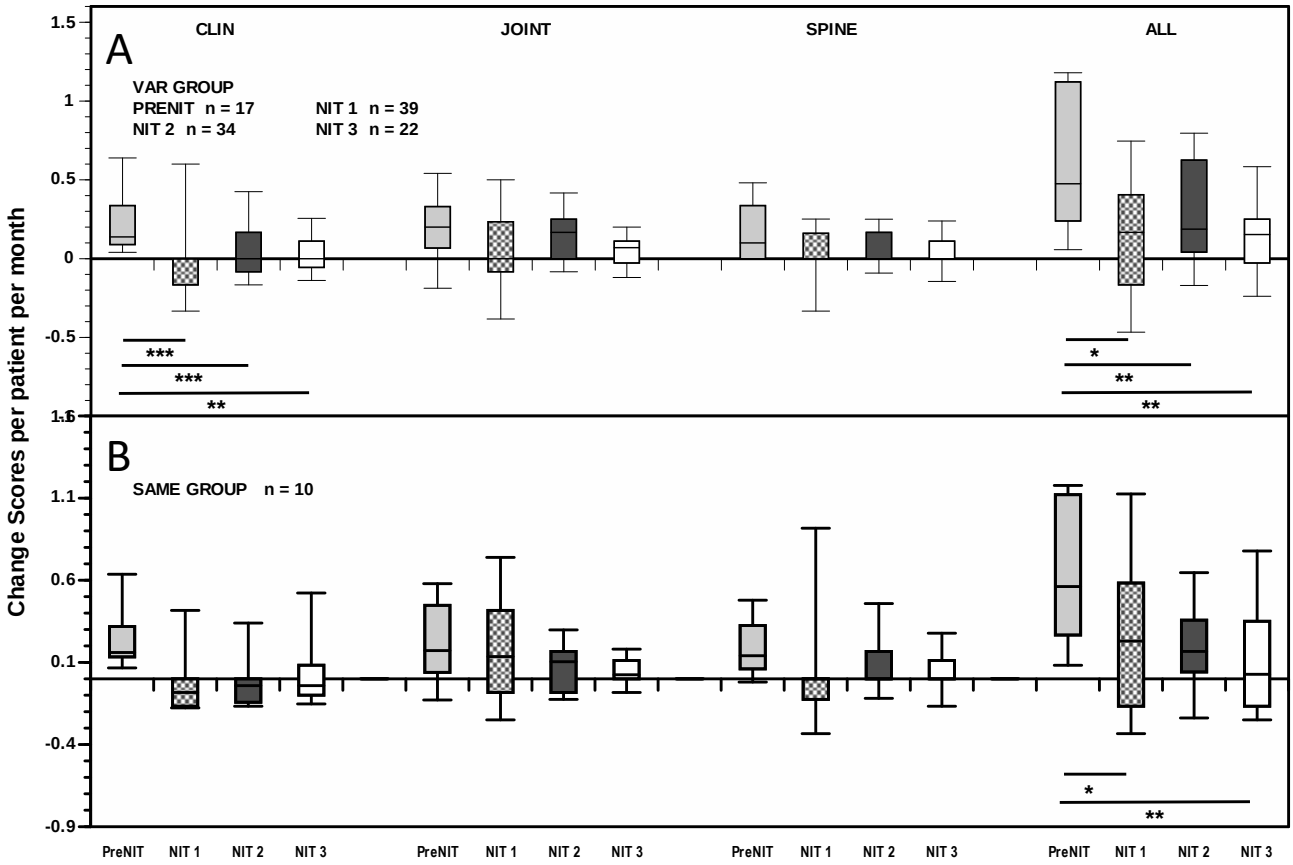

Figure 2. (**A**) Components of AKUSSI *Change* Scores per patient per month in SAME group (n=10). Scores are shown as boxplots with interquartile range. The levels of significance of results are shown as *p<0.05; **p<0.01; ***p<0.001. (**B**)**.** Components of AKUSSI *Change* Scores in VAR group (n variable). Scores are shown as boxplots with interquartile range. The levels of significance of results are shown as *p<0.05; **p<0.01. The score change tends to be slower following nitisinone usage both in the SAME and the VAR groups across CLIN, JOINT, SPINE and ALL categories.

VAR Group: At baseline the following numbers of events (in brackets) were observed in the VAR group: prostate stone (13), renal stones (6), osteopenia (27), fractures (21), tendon rupture (5), muscle rupture (5), ligament rupture (8), aortic sclerosis (14), aortic stenosis (6), hearing loss (11) and joint replacements (36) (Table S1). There was a significant increase (**Table 2**) in ALL category between V0 and V1, although a similar pattern was observed for other categories between V0 and V1. There was no evidence of differences for other comparisons including V2, V3 and V4.

VAR Group score change over time (Table 3; Figure 2b): The score change per patient per month showed lower values with NIT-1, NIT-2 and NIT-3 compared against PRE-NIT in almost all categories. Comparisons were significantly different (Table 3) between NIT-1 and PRE-NIT for ALL and ALL WITHOUT PAIN categories; similarly, significant differences were observed between NIT-3 against PRE-NIT for the ALL category. Change in combined ear and eye pigment scores was lower during nitisinone usage (Figure 3).

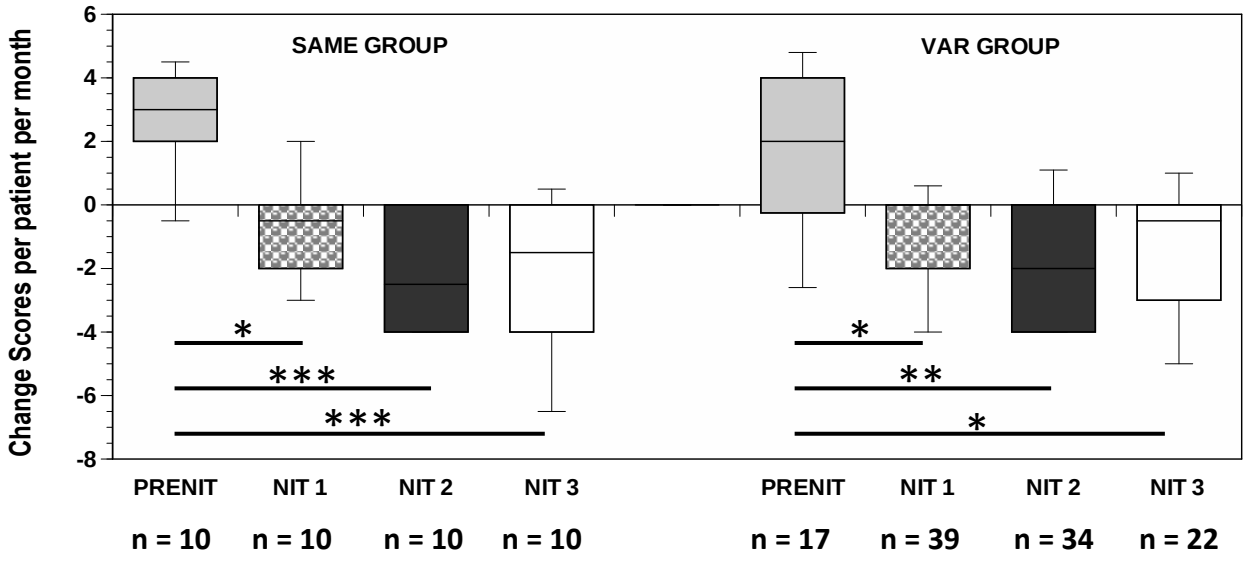

Figure 3. Change in combined eye and ear ochronosis scores per patient per month in SAME and VAR groups in the PRENIT, NIT 1, NIT 2 and NIT 3 categories. Significant decrease in rate of change seen following years of nitisinone usage (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Overall, the major finding is one of consistent change in CLIN, JOINT, JOINT NP, SPINE, SPINENP, ALL and ALLNP in the same direction indicating slower progression post-nitisinone in SAME and VAR groups. Changes in SAME and VAR groups (Table S1) have been described in detail. Despite high prevalence of disease features, due to numbers of patients not being large and due to the unpredictability of events, the incidence of new events such as fractures, ruptures, renal stones and joint replacements are low. The mean age of the groups was around 48 years in all categories and comparable in both the SAME and VAR groups.

Discussion

It is well established that nitisinone is effective in reducing circulating HGA in humans [6,8,13], and preventing ochronosis in AKU mice [9,10], but there is no data to date demonstrating a decrease in ochronosis or a reduction in the rate of disease progression in AKU patients. A previous nitisinone interventional clinical study of AKU patients was inconclusive for the primary outcome, namely range of motion of the hip joint, despite clear metabolic efficacy of nitisinone 2 mg decreasing daily urine HGA excretion by more than 95% [8].

Introne *et al* [8] compared the lateral rotation of the hip between the nitisinone treated group and the untreated group to conclude on clinical efficacy of nitisinone in AKU. Due to the ultra-rare nature of AKU, a disease with a frequency of 1 in 250,000 to 1 in 1,000,000, it would be difficult to have a sufficiently large number of patients to demonstrate a difference in a single end point such as lateral rotation of the hip [19]. Instead, analysis of the data carried out and presented in this manuscript from the NAC aimed to look at a composite end point.

The present data analysis is an audit of a service using nitisinone off-label. Our patient groups are highly comparable in terms of numbers of individuals and duration of nitisinone therapy to the NIH nitisinone intervention study, where 20 patients received nitisinone for three years and 20 did not [8]. Nitisinone is used in the NAC at the same dose as in the NIH nitisinone interventional study. By calculating the difference in scores between V0 and V1 (PRENIT), it was possible to carry out the assessment of disease progression in both SAME and VAR groups over 3 years without treatment with nitisinone, which is similar to the no-treatment group in the NIH study.

Nitisinone decreased urine HGA by greater than 80% in both the SAME and the VAR groups (Fig S2). This was only slightly less than in the nitisinone intervention study [8], and is reasonable considering that the present data analysis is from a service evaluation rather than a research study. The magnitude of reduction in HGA needed for optimal benefit, thus the dose of nitisinone needed to make a clinical difference, is not currently known.

In the PRENIT score change analysis, the major finding is the consistent increase in the AKUSSI between the V0 to V1 comparisons across all categories. This suggests progression of AKU in patients who are nitisinone-naive. It is noteworthy that none of the other AKUSSI categories showed a significant difference at any follow-up visits once patients started treatment with nitisinone, both in the SAME and the VAR groups. This suggests that nitisinone 2 mg daily is having a similar effect in slowing the progression of AKU both in the SAME and the VAR groups. This is further supported by

the finding of a significant and progressively slower rate of AKUSSI score change per patient per month while on treatment with nitisinone, with the longer the duration of nitisinone usage showing greater decrease in rate of change per patient per month, both in the SAME and the VAR groups (Figure 2a, b). The change in combined ear and eye ochronosis scores were lower post-nitisinone both in the SAME and the VAR groups (Figure 3). This demonstrates, for the first time in humans, that the process of ochronosis is retarded by nitisinone, as it has been shown to do in AKU mice [9,10].

The differences in ALL AKUSSI scores between the V0 and V1 in both the SAME ($p<0.01$) and the VAR ($p<0.05$) groups were significant. To translate these changes in terms of clinical significance, the total score change between the V0 and V1 score in the SAME and VAR groups, were respectively 224 and 278.8, hypothetically equivalent to 56 (5.6 per patient) and 69.7 (4.1 per patient) joint replacements [or 18.7 (1.87 per patient) and 23.2 (1.36 per patient) severe cases of aortic stenosis or other disease feature equivalents] without nitisinone treatment. Following 3 years of treatment with nitisinone the total score change between the V0 and V1 in the SAME and VAR groups were respectively 54 and 123.2, hypothetically equivalent to 13.5 (1.35 per patient) and 30.8 (1.81 per patient) joint replacements [or 4.5 (0.45 per patient) and 10.3 (0.61 per patient) cases of severe aortic stenosis or other disease feature equivalents] respectively. To capture the number of joint replacements in the range of 56 and 69.7, would require an enormous number of AKU patients, not feasible in an ultra-rare disease. However, a significant change in the composite AKUSSI score, which reflects pathology based on a common mechanism namely HGA-related ochronosis, allows for a better assessment of efficacy of treatments such as nitisinone.

In the SAME group, the events leading to the CLIN score changes between V0 and V1 is shown (numbers of each event in bracket) (Table S1). The change in CLIN features at each visit in the SAME and VAR groups are also shown. These highlight the high prevalence of features in a slowly progressive condition, where the relatively lower incidence of these features, highlight the difficulty of using a

single feature to determine outcomes, and thus supporting the use of a composite score approach to evaluate efficacy of therapy.

HGA by itself causes features of AKU such as lithiasis. Nitisinone by decreasing HGA should prevent HGA stone formation. In addition, nitisinone should decrease the formation of ochronotic pigment by decreasing HGA and thereby decrease morbidity. The data from the SAME and VAR groups, showing that the eye and ear pigment changes were stabilised, strongly suggest this is the case.

The AKUSSI is a semi-quantitative tool based on categorical and partially continuous scoring of key features of AKU. The AKUSSI employed in the data analysis presented in this manuscript has been adapted from the one previously published [15,16], by removal of less specific disease features such as congestive heart failure, Parkinson's disease, strokes and electrocardiographic abnormalities. The ocular ochronosis assessment has been improved. Most of the scores in the AKUSSI were objective based on investigations, although there were a few subjective features such as joint pains.

As these data were obtained as part of service delivery assessment, there are inevitable limitations. All patients were eligible to receive nitisinone and therefore there was no randomization of treatment intervention. However, a group of patients were followed up for a near-equal duration prior to and following nitisinone treatment, minimising bias. The NAC patients also received individually tailored treatments such as dietary advice, physiotherapy, analgesia and possibly nerve blocks and there is a theoretical but unlikely possibility that these additional treatments could have led to the improvements observed. This is why JOINT, SPINE and ALL AKUSSI were also calculated without pain scores as in JOINT NP, SPINE NP and ALL NP AKUSSI scores (Table 2 and 3). It is noteworthy that despite removal of pain scores the statistical significance of results remained relatively unaffected. It is also unlikely that any interventions for pain relief carried out in the NAC visit lasted a full year in this annual service visit model. It should be noted that relieving pain might also have adversely affected outcomes, as pain is an important defence against unnecessary usage of a damaged tissue, allowing the affected tissue to rest, heal and repair itself. The ochronotic tissue in AKU is brittle and breaks down easily [20]

and any treatment that does not address the underlying pathophysiology is likely to be palliative at best and harmful at worst. All patients were also free to receive further treatments as needed locally after their NAC visit, both before and during nitisinone therapy. For all these reasons we believe that treating analgesia in the NAC was not a confounder in the analysis of results.

In the NAC, lower protein diet was employed in managing tyrosinaemia after nitisinone therapy. However, there is little reliable literature attesting to efficacy of diet in modifying AKU especially in adults [8]. Physiotherapy like analgesia is palliative at best and should not modify ochronosis as there is no plausible mechanism to alter disease progression.

There were no HGA measurements in urine or serum from the V0 visits as samples were not acid stabilised and stored. However, we do not believe that this is a serious failing as the literature on metabolic efficacy is overwhelmingly established and all patients had proven AKU.

Despite these limitations, the data show that 2 mg of daily nitisinone decreases the clinical progression of AKU over the three-year period. We have been able to show for the first time that nitisinone not only decreases HGA in AKU, but also reduces the rate of progression of what is believed to be an irreversible disease [10]. We have also shown for the first time that nitisinone arrests ochronosis, the cause of tissue damage in AKU.

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