Efficacy and safety of once-daily nitisinone for patients with alkaptonuria (SONIA 2): an international, multicentre, open-label, randomised controlled trial

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Suitability of Nitisinone in Alkaptonuria 2 (SONIA 2) - An international, multicentre, randomised, evaluator-blinded, no-treatment controlled, parallel-group study to assess the efficacy and safety of once daily nitisinone in patients with alkaptonuria after 12 months of treatment, followed by an additional 36-month treatment period

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Corresponding Author:	Lakshminarayan R Ranganath, MBBS Royal Liverpool University Hospital Liverpool, Merseyside UNITED KINGDOM
First Author:	Lakshminarayan R Ranganath, MBBS
Order of Authors:	Lakshminarayan R Ranganath, MBBS
	Eftychia Eirini Psarelli, Ptychion, MSc
	Jean-Baptiste Arnoux, MD
	Daniela Braconi, PhD
	Michael Clarke Briggs, M.B.,Ch.B F.R.C.S.(Ed, Ophth.)
	Anders Bröijersén, MD, PhD
	Nadia Burgess, BSc
	Helen Bygott, BSc
	Trevor Frederick Cox, BSc, MSc, PhD
	Andrew Stuart Davison, BSc MSc MSc FRCPath
	Jane P Dillon, MSc
	Michael Fisher, BSc (hons), MBChB, PhD
	Richard FitzGerald, MBBS, FRCP
	Federica Genovese, PhD
	Helena Glasova, MD, PhD
	Anthony Kent Hall, MB BS BSc AKC
	Andrew Thomas Hughes, BSc(Hons), MPhil
	Juliette Holly Hughes, MBiolSci
	Richard Imrich, MD, PhD, DSc
	Jonathan Charles Jarvis, BSc, PhD
	Milad Khedr, MBBS, MRCP, MRCGP, FRCPath
	Dinny Laan, MSc
	Kim-Hanh LE QUAN SANG, PhD-Chemistry & PhD-Clinical Pharmacology
	Emily Luangrath, MSc
	Oľga Lukáčová, MD, PhD
	Anna Margaret Milan, BSc(Hons), MSc, PhD
	Alpesh Mistry, FRCR

	Vanda Mlynáriková, MD, PhD
	Brendan Paul Norman, BSc (Hons), MSc, PhD
	Birgitta Olsson, MSc
	Nicholas Peter Rhodes, BSc, MSc, PhD
	Jozef Rovenský, MD, DSc, FRCP
	Mattias Rudebeck, MSc BMedSc
	Annalisa Santucci, PhD
	Ella Shweihdi, MSc
	Ciaran Scott, BSc
	Jana Sedláková, MD, PhD
	Nicolas Thierry Sireau, MA (Oxon), MSC, PhD
	Roman Stančík, MD, PhD
	Johan Szamosi, MSc
	Sophie Taylor, MSc
	Christa Irene van Kan, MSc
	Sobhan Vinjamuri, MD MSc FRCP
	Eva Vrtíková, MD
	Christopher Webb, FRCS
	Elizabeth Alexandra West, MBChB MRCP UK
	Elizabeth Záňová, MD, PhD
	Andrea Zatkova, PhD
	James A Gallagher, PhD
Manuscript Region of Origin:	UNITED KINGDOM
Abstract:	Alkaptonuria (AKU) is a genetic, rare, multisystem disease, characterised by accumulation of homogentisic acid (HGA). There is no approved HGA-lowering therapy. Nitisinone decreases HGA generation. SONIA 2 investigated the effect of nitisinone on the disease process, and on progression of AKU. Methods: This was a 48-month randomised, open-label, evaluator-blinded, parallel-group study performed in the UK, France and Slovakia. Patients ≥25 years of age with confirmed AKU and any clinical disease manifestations were randomised to receive either 10 mg nitisinone or no treatment. Site visits were performed at 3 months and yearly thereafter. Results from history, photographs of eyes/ears, whole body scintigraphy, echocardiography, and abdomen/pelvis ultrasonography, were combined to derive the Alkaptonuria Severity Score Index (cAKUSSI). The primary objective was to show decrease in daily urinary HGA (u-HGA 24) excretion at month 12. Secondary objectives included comparing clinical outcomes after 48 months. Findings: 69 patients were randomised to nitisinone and 69 to the control group. 55 patients in the nitisinone group and 53 in the control group completed the study. u-HGA 24 at 12 months was statistically significantly decreased by 99.7% in the nitisinone group compared with control. The adjusted geometric mean (ratio nitisinone/control) was 0·003 (0·003 – 0·004), p<0·0001. cAKUSSI scores decreased statistically significantly at 48 months in nitisinone group compared with control [adjusted mean difference -8·6 (-16·0 – 1·2), p=0·02]. The incidence of adverse events (AEs) was similar for the groups, but numerically more AEs were reported in the nitisinone group (400 AEs in 85·5% of patients) versus control (284 AEs in 82·6% of patients). Interpretation: Nitisinone 10 mg daily was well tolerated and effective to reduce urinary excretion of HGA. Nitisinone decreased ochronosis and improved clinical signs, indicating a slower disease progression. Funding

	European Commission Seventh Framework Programme funding was granted in 2012 (DevelopAKUre, project number: 304985).

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Ranganath LR[1,8], Psarelli EE[2], Arnoux JB[3], Braconi D[4], Briggs M[5], Bröijersen A[6], Loftus N[7], Bygott H[1], Cox TF[2], Davison AS[1], Dillon JP[8], Fisher M[9], Fitzgerald R[10], Genovese F[11], Glasova H[12,13], Hall AK[14], Hughes AT[1], Hughes JH[8], Imrich R[12,15], Jarvis JC[16], Khedr M[1], Laan D[17], Le Quan-Sang KH[3], Luangrath E[1], Lukáčová O[15], Milan AM[1], Mistry A[18], Mlynáriková V[15], Norman BP[8], Olsson B[6], Rhodes NP[8], Rovensky J[15], Rudebeck M[6], Santucci A[4], Shweihdi E[1], Scott C[19], Sedláková J[15], Sireau N[19], Stančík R[15], Szamosi J[6], Taylor S[7], van Kan C[17], Vinjamuri S[20], Vrtíková E[15], Webb C[21], West E[22], Záňová E[15], Zaťková A[12], Gallagher JA[8]

[1]Departments of Clinical Biochemistry and Metabolic Medicine, Liverpool University Hospitals NHS Foundation Trust (LUH), Prescot Street, Liverpool, L7 8XP, UK; [2] Liverpool Clinical Trials Centre, University of Liverpool, Block C, Waterhouse Building, Liverpool L69 3GL, UK; [3]Hôpital Necker-Enfants Malades, Paris Cedex 15, France; [4]Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Siena, Italy; [5]Department of Ophthalmology, LUH; [6]Swedish Orphan Biovitrum AB (publ), Stockholm, Sweden; [7]Department of Physiotherapy, LUH; [8]William Henry Duncan Building, West Derby Street, Liverpool, L7 8TX; [9]Department of Cardiology, LUH; [10]Department of Clinical Pharmacology, LUH; [11]Nordic Bioscience, Herlev, Denmark; [12]Institute of Clinical and Translational Research, Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovakia; [13]Institute of Pharmacology and Clinical Pharmacology, Slovak Medical University, Bratislava, Slovakia; [14]Cudos BV, Hoofddorp, Netherlands; [15]National Institute of Rheumatic Diseases, Piešťany, Slovakia; [16]School of Sport and Exercise Science, Liverpool John Moores University, Liverpool, UK; [17]PSR group B.V., Hoofddorp, Netherlands; [18]Department of Radiology, LUH; [19]AKU Society, Cambridge, UK; [20]Department of Nuclear Medicine, LUH; [21]Department of ENT, LUH; [22]Department of Dermatology, LUH.

Corresponding author: LR Ranganath, Department of Clinical Biochemistry and Metabolic Medicine, Royal Liverpool University Hospital, Prescot Street, Liverpool, L7 8XP; e-mail: lrang@liv.ac.uk

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Abstract

Alkaptonuria (AKU) is a genetic, rare, multisystem disease, characterised by accumulation of homogentisic acid (HGA). There is no approved HGA-lowering therapy. Nitisinone decreases HGA generation. SONIA 2 investigated the effect of nitisinone on the disease process, and on progression of AKU.

Methods: This was a 48-month randomised, open-label, evaluator-blinded, parallel-group study performed in the UK, France and Slovakia. Patients ≥25 years of age with confirmed AKU and any clinical disease manifestations were randomised to receive either 10 mg nitisinone or no treatment. Site visits were performed at 3 months and yearly thereafter. Results from history, photographs of eyes/ears, whole body scintigraphy, echocardiography, and abdomen/pelvis ultrasonography, were combined to derive the Alkaptonuria Severity Score Index (cAKUSSI). The primary objective was to show decrease in daily urinary HGA (u-HGA_{24}) excretion at month 12. Secondary objectives included comparing clinical outcomes after 48 months.

Findings: 69 patients were randomised to nitisinone and 69 to the control group. 55 patients in the nitisinone group and 53 in the control group completed the study. u-HGA_{24} at 12 months was statistically significantly decreased by 99.7% in the nitisinone group compared with control. The adjusted geometric mean (ratio nitisinone/control) was 0·003 (0·003 – 0·004), p<0·0001. cAKUSSI scores decreased statistically significantly at 48 months in nitisinone group compared with control [adjusted mean difference -8·6 (-16·0 – 1·2), p=0·02]. The incidence of adverse events (AEs) was similar for the groups, but numerically more AEs were reported in the nitisinone group (400 AEs in 85·5% of patients) versus control (284 AEs in 82·6% of patients).

Interpretation: Nitisinone 10 mg daily was well tolerated and effective to reduce urinary excretion of HGA. Nitisinone decreased ochronosis and improved clinical signs, indicating a slower disease progression.

Funding

European Commission Seventh Framework Programme funding was granted in 2012 (DevelopAKUre, project number: 304985).

Research in context

Evidence before this study

There has been only one previous long-term clinical study using the potentially disease-modifying agent, nitisinone, to evaluate the effect of the drug on AKU disease progression (Medline search up to and including February 2020). The terms used in the Medline search were nitisinone, alkaptonuria, and outcomes. In addition, because AKU is a rare disease, personal contacts with researchers and clinicians in the field enable us to confidently state that there has been only one previous outcomes trial using nitisinone in AKU. The National Institutes of Health (NIH, USA) nitisinone outcomes study on 20 nitisinone-treated and 20 control AKU patients, employed an improvement in the lateral rotation of the hip as the endpoint to decide on efficacy of 2 mg nitisinone daily over 3 years, the effect on this endpoint deemed inconclusive. There have been three short-term studies, two in the NIH, USA, and one in Liverpool, UK, that reported the metabolic efficacy of nitisinone in terms of lowering HGA. An audit of the use of nitisinone 2mg daily off-label in the National AKU Centre in Liverpool, funded by NHS England Highly Specialized Services, showed metabolic benefit, arrest of ochronosis, and slower progression of AKU disease, but this was an audit of a service rather than a research study.

Added value of this study

The present international, multicentre, randomised, controlled, evaluator-blinded, parallel-group study is an analysis of the efficacy of using 10 mg nitisinone daily in AKU. Both the nitisinone-treated and control groups had similar numbers of patients. The outcome was based on the effect of nitisinone on the change in AKU severity score index, a composite disease score, over four years. The power of the study was much increased by the use of the composite score, AKU severity score index. The composite disease score outcome was also clear cut in showing that nitisinone decreased the progression of AKU for the very first time in a randomised study. The study of the control group over four years has improved our understanding of the natural history further.

Implications of all the available evidence

We believe that the publishing of our manuscript will provide a major boost to the study of, and progress in, rare diseases. Our manuscript will be of interest to the general readership of the journal because AKU is an iconic disease whose study foreshadowed genomic medicine. AE Garrod applied Mendel's Laws of inheritance to human disease as early as 1902 in his studies of AKU. AKU has the added attribute of being a rare disease, in which the natural history is incompletely understood (an attribute it shares with most rare diseases); rare disease is 'common' in the sense that virtually all readers will need to manage rare diseases, with lessons to learn from our experience. The lack of patient numbers in which to study and carry out fully powered clinical trials, as in major frontline diseases like cardiovascular disease and diabetes, challenges the rare disease community to develop innovative ways to develop much needed therapies. We have employed a weighted composite score, which enabled us to overcome the low patient numbers by increasing power, and showing for the first time that nitisinone has disease-modifying attributes as well as metabolic efficacy. Our experience also

provides a powerful example of the effective re-purposing of an existing drug for a novel indication, which may be a more practical strategy than developing entirely new drugs for rare diseases. Our experience may empower researchers into the other 7000 rare diseases in their approach to achieve solutions in their diseases of interest. Finally, our findings demonstrate for the first time the efficacy of a disease modifying drug in the iconic disease AKU, and therefore bring hope to patients with this condition.

Background

Alkaptonuria (AKU) (OMIM 203500) is a rare, serious, autosomal recessive multisystem disorder[1] affecting approximately one in every 250 000 to 1 million people.[2] The disease was the first ever described in a paper by AE Garrod in 1902[3], in which Mendel's laws of inheritance were applied in human disease. Still, AKU lacks a pharmacological treatment. Genetic deficiency of homogentisate dioxygenase activity (HGD) results in accumulation of homogentisic acid (HGA) (Figure S1). HGA is then progressively deposited as yellow/dark pigment in connective tissue, rendering these more rigid and eventually brittle, and prone to degradation, a process termed ochronosis.[4,5] As the causal agent, HGA may therefore represent a suitable surrogate for a clinically meaningful endpoint in clinical trials. This was also suggested by the European Medicines Agency (EMA), during scientific advice before starting our clinical program. Degradation of ochronotic tissue is mainly responsible for the multisystem involvement, with varying phenotype, characterised by severe premature spondyloarthritis, lithiasis, cardiac valve disease, fractures, muscle and tendon ruptures, and osteopenia.[6,7] Palliative analgesia and arthroplasty is the mainstay of AKU therapy.

AKU is a disorder of tyrosine metabolism like another inherited condition known as hereditary tyrosinaemia type 1 (HT-1) (OMIM 276700). In HT-1, there is a deficiency of fumarylacetoacetate hydrolase, resulting in early liver and kidney disease and death in childhood if untreated.[8,9] Nitisinone (2-[2-nitro-4-(trifluoromethyl) benzoyl] cyclohexane-1,3-dione) is an inhibitor of the hydroxyphenylpyruvate dioxygenase (HPPD) (EC 1.13.11.27) and has been used in HT-1 since 1991. As activity of HPPD leads to formation of HGA (Figure S1) nitisinone was hypothesised in the late 1990s to be a potential treatment for AKU.[10] Following initial research of nitisinone for treatment of AKU[1,11], a three-year clinical trial comparing a nitisinone-treated patient group, receiving a 2 mg daily dose, with an untreated group, with 20 AKU patients in each group, was reported as inconclusive, despite showing excellent biochemical efficacy.[12]

Despite this setback, research into the use of nitisinone in AKU has continued. In addition, nitisinone 2 mg daily has been reimbursed for use in the United Kingdom's National Alkaptonuria Centre (NAC) since 2012, and a recent publication described positive outcomes for nitisinone in its metabolic and non-metabolic effects.[13,14] However, the off-label use in the NAC is, despite collecting high-quality data in a protocolised manner, in a service capacity and not a controlled clinical trial.

In designing the SONIA 2 study, it was assumed that the NIH trial did not succeed because of the small number of patients recruited, insufficient duration in such a very slowly progressive condition as AKU, the incomplete understanding of the natural history, and use of a single and possibly unreliable outcome measure in this multifaceted disease. An identification campaign to maximise patient recruitment for a new trial was subsequently carried out both in the UK and the rest of Europe.[15] A better understanding of the natural history and it's modification by nitisinone was shown in a mouse AKU model.[16-18] Careful phenotyping of the disease in a cohort of untreated AKU patients resulted in a composite score, termed AKU Severity Score Index (AKUSSI), a key factor when researching a multifaceted condition with variable phenotype.[19,20] In addition, a new clinical trial of nitisinone, with a considerably higher number of patients and a longer duration, was considered because of the naturally slow progression. The dose used in the inconclusive NIH trial was based on the experience of administering nitisinone to two AKU patients[10]; further, the EMA suggested finding a dose that normalises HGA, and therefore the issue of optimal dose was revisited in a dose-response study, the 'Suitability Of Nitisinone In Alkaptonuria 1' (SONIA 1).[21] In that study, the 8mg daily dose of nitisinone resulted in a mean reduction of u-HGA_{24} of 98.8 %, with a clear dose-response and much less variability compared with the other doses studied (1, 2 and 4 mg). An increase in tyrosine levels was seen at all doses but the dose-response relationship was less clear, with no tyrosine-related adverse events seen at any dose. Since the 8mg dose resulted in u-HGA_{24} close to normal values, a dose of 10 mg daily, which was achieved with an available capsule strength, was selected for the new trial, SONIA 2. All of these factors influenced the design of SONIA 2 in which the safety and efficacy of nitisinone 10 mg daily in AKU was investigated.

METHODS (additional details available in supplementary material)

Objectives

The primary objective in SONIA 2 was to demonstrate that nitisinone was superior compared to control in reducing u-HGA_{24} in patients with AKU after 12 months. Secondary objectives were defined to demonstrate the sustained control of urinary and serum HGA up to 48 months and to demonstrate the effect on clinical parameters and to assesses the safety of nitisinone in AKU.

Study design

SONIA 2 was a four-year, open-label, evaluator-blinded, multicentre, randomised, no-treatment controlled, parallel-group study. A formal interim analysis was planned when all patients completed 12 months of treatment. This analysis included the complete set of efficacy and safety data up to 12 months, thus including the final analysis of the primary endpoint. The purpose was to evaluate if data demonstrated results suitable for a regulatory application already at that stage, even though the study was to continue for another 3 years to collect more complete efficacy and safety data. The study design is summarised in Figure S2.

The study was performed at three investigational sites: Liverpool (UK), Paris (France) and Piešťany (Slovakia). Independent Ethics Committee at each centre approved the study.

Patients

The aim was to recruit 140 patients aged 25 years or older, with a confirmed diagnosis of AKU and any clinical manifestation in addition to increased HGA; 70 randomised to nitisinone 10 mg and 70 to a control (no-nitisinone) group. All patients provided written informed consent prior to inclusion.

Treatment

Oral nitisinone (Orfadin®) 10 mg daily was administered in the treated group. The control group did not receive the study drug.

Nitisinone was withdrawn in patients who developed signs of ocular tyrosine-related adverse events (AEs). If feasible, once the symptoms had resolved (minimum 2 months after temporary withdrawal), nitisinone was reintroduced at a lower dose (2 mg daily). Alternatively, the patient was withdrawn from the study. If ocular tyrosine-related symptoms reappeared on the lower dose, nitisinone was permanently withdrawn and the patient was monitored until the symptoms resolved.

There were no restrictions regarding concomitant medications. Patients in both groups could freely use e.g. analgesics, anti-inflammatory drugs and others as needed to treat symptoms of AKU.

Randomisation and masking

Patients were randomly assigned to one of the two groups in a 1:1 ratio. The randomisation was stratified by study centre and age (≤ 55 years and > 55 years) and was carried out by using

randomly permuted blocks (4 patients/block) within each study centre and age stratum. The study statistician created a program to randomly assign the patients to the two treatment groups using the SAS System. The randomisation was centrally implemented in the electronic CRF system (Viedoc®).

It is not possible to blind a study with nitisinone in AKU because one of the signs of the disease is that the urine darkens due to oxidation of excreted HGA. Patients can therefore easily notice if they are receiving active drug or not. Therefore, the control group received no placebo treatment. Instead, the study was evaluator-blinded as far as possible. Assessments which did not require direct contact between the evaluator and the patient (such as evaluation of images) were blinded during the entire study. The blinded evaluators were experts in their respective field, and never met the patients. Other assessments were made by objective measurements (Table S1), such as that of bone density. It is, however, recognised, that reporting of subjective assessments may have introduced bias for some of the secondary endpoints, such as pain and quality-of-life assessments, and reporting of adverse events.

Procedures

In addition to a 24-h urine (u-HGA_{24}) collected into acid for HGA and creatinine determination, fasting acidified serum for HGA, tyrosine and creatinine, a number of assessments and investigations were carried out (supplementary material). These included collection of medical history and physical examination, including those specific for AKU, a wide range of clinical outcome measures, including range of motion tests and quality of life assessments, safety assessment and other procedures shown in Table S1 and elsewhere.[13,14]

AKU Severity Score Index (AKUSSI) assessments (Table S1)

The AKUSSI incorporates multiple, clinically meaningful AKU outcomes that can be described in a single score.[13,19,20] All items included in the AKUSSI were assessed at baseline and yearly thereafter. Two types of AKUSSI were included as secondary outcomes in SONIA 2. These are the clinical evaluation AKUSSI (cAKUSSI) and a modified AKUSSI (mAKUSSI = cAKUSSI without pigmentation features).

Patients visited study sites at 3 months, and then annually up to month 48; a close-out phone call took place at month 49. A questionnaire, completed by patients, collected safety information at 6, 18, 30 and 42 months.

At each visit, AEs and laboratory values were recorded. AEs included clinically significant signs and symptoms and abnormal test findings (e.g. laboratory analysis results, vital signs or ECG) that the investigator considered clinically significant and/or that led to a medical/surgical intervention including withdrawal of nitisinone or discontinuation from the study.

Statistical analysis

Only a few subjects would be needed to detect a statistically significant effect on the primary endpoint, u-HGA_{24}. Therefore, the sample size was based on the AKUSSI score, to allow the possibility to establish an effect on a clinical endpoint. Using data from a cross-sectional study of AKU using AKUSSI[13,14,20] and follow-up data, it was assumed that if nitisinone reduced the mean increase in AKUSSI over the 4-year period to 4 points, compared to 8 points in the control group, and taking the standard deviation of the increase to be 8, then a sample size of 64 per group was required for a two-sided t-test with 80% power for a significance level 0·05. With an estimated 10% drop-out rate, a sample size of 70 per group was required (140 patients in all).

The Full Analysis Set (FAS) including all randomised patients was used for the analysis of efficacy variables, containing all randomised patients who had a valid u-HGA_{24} at baseline.

The Safety Analysis Set was used for the analysis of safety variables. All randomised patients were included in both sets.

All statistical analyses were performed with the SAS System (version 9.3, SAS Institute, Cary, NC). Two-sided 95% confidence intervals corresponding to a two-sided 5% level of significance were used throughout the analyses. All relevant study data were tabulated with descriptive statistics, including mean, standard deviation, standard error of the mean, median, minimum and maximum for the continuous variables, and frequencies and proportions for the categorical variables. Both absolute values and changes from baseline were tabulated, if feasible. No allowance for multiplicity was made.

Analysis of primary endpoint

The primary endpoint was the u-HGA_{24} in patients with AKU after 12 months.

A longitudinal model (mixed model for repeated measures (MMRM) with an underlying normal distribution was fitted for the analysis of the primary endpoint. An unstructured covariance matrix was used along with a restricted maximum likelihood method (REML),

while the degrees of freedom were estimated using Kenwood-Rogers method. Treatment, site, age category, visit and treatment by visit interaction were added as fixed factors in the model together with the baseline log(u-HGA_{24}) value as a covariate and with subject-within-site included as a random factor. The analysis was performed using the log(u-HGA_{24}) as dependent variable. Model based point estimates and associated two-sided 95% confidence intervals were calculated.

Analysis of secondary endpoints supporting primary endpoint

u-HGA_{24} at month 3, 24, 36 and 48 was analysed using the same MMRM model as in the primary endpoint analysis.

Analyses of other endpoints

Changes from baseline in cAKUSSI, mAKUSSI, individual AKUSSI items, and pre-dose s-HGA, were also analysed. For continuous secondary endpoints, the same statistical model as in the primary endpoint analysis was used, with the exception that these analyses were conducted on the original scale without transformation. However, this was not the case for s-HGA and s-Tyr where log transformation was used. Ordinal secondary endpoints were modelled using a generalised estimating equations (GEE) approach, whereas count data was modelled using an MMRM with an underlying Poisson distribution.

Analysis of safety data

All adverse events (AEs) during the study were coded using the Medical Dictionary for Regulatory Activities (MedDRAv.16.0). The incidence of AEs was summarised in frequency tables. The changes in safety laboratory parameters from baseline to all post-baseline visits were summarised by treatment group and visit using descriptive statistics. These included serum concentration of clinical chemistry, haematology, vital signs, electrocardiogram (ECG) and corneal eye assessments.

A Data Monitoring Committee was assigned to safeguard the interests of study participants and to continuously monitor the safety of the patients in the study.

The study was registered at clinicaltrials.gov (NCT01916382).

Role of the Funding Source

This study was funded by a grant from the European Union Framework Programme 7 (DevelopAKUre, project number: 304985). The funder of the study had no role in study design,

data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Disposition of patients: 139 patients were screened and 138 included the study between 7th May 2014 and 16th February 2015, with 69 patients randomised to each of the two study groups. First patient was randomized on May 7, 2014 and the last patient's last visit was February 15, 2019. SONIA 2 was funded by the European Commission under their FP 7 programme, with a strict time limit for its completion. Therefore, as the number of recruited patients was deemed sufficient at the end of the recruitment period, recruitment was ended after 138 patients were included (139 screened), to meet these timelines. Of these, 108 patients completed the study. All 138 patients (69 per group) were included in the analysis and this was by originally assigned groups. The main reason for discontinuation in the control group was withdrawn consent (10 patients), while AEs were the most common reason for withdrawal (nine patients) in the nitisinone group (Figure 1).

Demographic data and other baseline characteristics: The two groups were well balanced. The majority of the patients (134 patients, 97·1%) were Caucasian. There were more males in the nitisinone-treated group (45 patients, 65·2%) compared to the control group (40 patients, 58·0%). The mean age was slightly lower in the control group compared to the nitisinone group (Table 1).

Efficacy and safety assessments

Primary outcome - Urinary HGA: The u-HGA_{24} was statistically significantly decreased in the nitisinone-treated group compared to the control at all visits after baseline. These findings were consistent irrespective of age, sex, or study site. At month 12, the time of evaluation of the primary endpoint, the adjusted mean u-HGA_{24} had statistically significantly decreased by 99·7% in the nitisinone group compared to the control group [adjusted geometric mean (ratio nitisinone/control) 0·003 (0·003 – 0·004), $p<0·0001$] (Table 2, Figure 2A).

Serum HGA: At baseline, the geometric mean s-HGA was comparable for the two study arms. At month 12, the adjusted geometric mean s-HGA in the nitisinone group had statistically significantly decreased by 98·8% compared to the control group [adjusted geometric mean (ratio nitisinone/control) 0·01 (0·01 – 0·02)]. At each visit after baseline, the difference in

change from baseline in s-HGA between the study arms was statistically significant (p<0·0001) (Table 2, Figure 2B).

Interim analysis of secondary efficacy outcomes: The 12-month analysis of the secondary efficacy endpoints did not support a regulatory authority application for the new indication.

AKUSSI (cAKUSSI and mAKUSSI) assessments: At baseline, cAKUSSI was slightly higher in the nitisinone group than in the control group. Over time there was an increase in cAKUSSI in the control group from baseline to month 48, while there was less of an increase in the nitisinone group. The difference between the two groups in the change from baseline to month 48 was statistically significant [adjusted mean difference -8·6 (-16·0 – 1·2), p=0·02]. The adjusted mean increase was 15·1 and 6·7 points in the control and nitisinone groups respectively, over the duration of the study (Tables 2, S1; Figure 3A).

mAKUSSI: At month 48 there was no statistically significant difference between the two groups in change from baseline [adjusted mean difference -3·6 (-9·6 – 2·4), p=0·23] (Table 2). There was, however, a continuous increase in mAKUSSI in the control group from baseline to Month 48, while a slower increase was observed for the nitisinone group (Tables 2, & S1, Figure 3B).

Selected individual AKUSSI items

Statistically significant differences between the two treatment groups were observed at Month 48, and for some variables also from earlier time points, for the following variables.

- Eye pigmentation (Table 2, Figure S3A)
- Ear pigmentation (Table 2, Figure S3B)
- Osteopenia of the hip (T-scores for bone density) (Table 2, Figure S4A)
- Number of spinal regions with pain (Table 2, Figure S5B)

For the number of joints with pain, a statistically significant difference in favour of nitisinone was observed at Month 12 [adjusted mean difference -0·9 (-1·6 – -0·1), p=0·02]. Numerically, the difference between the groups was relatively constant at subsequent visits, and at Month 48 [adjusted mean difference -0·7 (-1·6 – 0·1), p=0·10].

An increasing gap, between the two treatment groups from baseline to Month 48, supporting a lower rate of disease progression in the nitisinone group, was observed for the following variables, however the result failed to reach statistical significance (p-values>0·05):

- mAKUSSI (Table 2, Figure 3B).
- Number of fractures (Table 2, Figure S4B).
- Number of tendon, ligament and muscle ruptures (Table 2, Figure S4C).

Other key secondary outcomes

Consistent trends towards better outcome in the nitisinone group compared to controls were also observed for:

- Quality of life (SF-36) (Figure S6).
- Self-evaluated transition (in SF-36) (Table S8).
- Range of motion of the joints (Figure S7).

No notable difference between the treatment groups was observed for any of the other variables.

Safety:

A total of 400 AEs in 85·5% of patients in the nitisinone group and 284 AEs in 82·6% of patients in the control group were reported. Most AEs reported were within the system organ class (SOC) "Musculoskeletal and connective tissue disorder" (mostly manifestations of AKU); 53 and 54 events were reported for 24 patients in the control group and 31 patients in the nitisinone group, respectively. "Infections and infestations" was the second most common SOC. There was a higher incidence of AEs in this SOC in the nitisinone group; 56 AEs were reported for 27 patients while in the control group there were 24 AEs reported for 11 patients. Pneumonia and bronchitis were more commonly reported in the nitisinone group than in the control group. Other than that, there was no clear pattern. "Eye disorders", the third most common SOC, were reported for 8 (11·6%) patients in the control group and 25 (36·2%) patients in the nitisinone group (Tables 3, S9, S10, S11, S12).

The incidence of AEs was 2·13 per 10 patient years in the control group and 2·27 in the nitisinone group. The incidence of eye-related AEs was 0·3 and 0·96 per 10 patient years respectively.

There were two deaths in the study, one due to heart failure and the other to myocardial infarction; both occurred in nitisinone-treated patients. None of the events was considered to be related to nitisinone treatment (Table 3).

A total of 53 patients, 26 in the control group and 27 in the nitisinone group, experienced at least one SAE during the study. None of these events was considered by the investigator to be related to nitisinone (Table S10). The SOC "Musculoskeletal and connective tissue disorders" had the highest number of SAEs, most of them related to joint replacements, fractures and other manifestations of AKU.

Ocular adverse events: A total of 77 AEs in the SOC "Eye disorders" were reported. In the control group, 8 (11·6%) patients reported 12 events. In the nitisinone group, 25 patients (36·2%) reported 65 events. A majority of these, such as keratopathy (10 patients), eye pain (8 patients), dry eye (6 patients), increased lacrimation (4 patients), ocular hyperaemia (4 patients), eye irritation (3 patients), are considered related to the increased levels of tyrosine caused by nitisinone treatment (Tables S9, S10).

Nine of the patients in the nitisinone group developed tyrosine-related keratopathy in one or both eyes confirmed by slit-lamp examination. One further patient, who could not come for a follow-up visit, was withdrawn due to suspected keratopathy based on convincing ocular symptoms. Of the nine keratopathy patients confirmed by slit-lamp examination, eight had other eye symptoms, such as pain, blurred vision or other signs. One patient reported no symptoms before keratopathy was seen by slit-lamp at a pre-planned visit. In these nine patients with keratopathy, complete resolution was shown at a follow-up visit at least 2 months after nitisinone withdrawal. Eight patients restarted nitisinone at a dose of 2 mg/day after the recovery; five of those had recurrent symptoms while three were still asymptomatic at the end of the study (Tables 3, S9).

As expected, serum tyrosine (s-Tyr) concentrations were above 500 µmol/L in all nitisinone-treated patients. At Month 12, the median value was 925 µmol/L, with a range from 563 to 1530 µmol/L. Decreasing the dose in those who switched from 10 to 2 mg following keratopathy had a limited effect on s-Tyr, with all patients still having levels above 500 µmol/L (Table S9, Figure S8).

DISCUSSION

The direct cause of morbidity in AKU is HGA accumulation, resulting from genetic HGD deficiency.[22] HGA is therefore a surrogate for a clinically meaningful endpoint in clinical trials.

In SONIA 2, u-HGA_{24} decreased markedly in the nitisinone-treated group compared to controls at all visits after baseline, and the primary objective of the study was thus met. Nitisinone efficiently decreased both u-HGA_{24} and s-HGA in nitisinone-treated patients, with mean values at month 12 decreasing by greater than 98% compared to control for both variables.

The difference between the groups in change in pigmentation, i.e. the ochronosis, which is the fundamental patho-physiological process in AKU, was statistically significant. This indicates that treatment with nitisinone arrested the ochronosis process in the eye and reversed it in the ear, by decreasing the accumulation of HGA. The crucial importance of ochronosis in AKU has recently been highlighted.[22] Reversal of the disease process in the ear was seen soon after starting nitisinone, and continued throughout the study duration. Although reversal of ochronosis in the ear was observed, the decrease in pigmentation was not total, and it is not clear whether a longer follow-up period would have shown more de-pigmentation.

A weighted composite score, the cAKUSSI, was used in SONIA 2, as for previously published data in AKU[13,13] This score was employed as it would have been 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 as employed in the NIH trial[12], given the ultra-rare nature of AKU, and the heterogeneous phenotypic severity. In SONIA 2, the baseline cAKUSSI scores were higher in the nitisinone group than in the controls. This may be because the nitisinone group was older, with an age difference in medians of three years, and containing more male patients, who have been shown to experience a more severe disease.[19,20]

In SONIA 2 a statistically significant effect (difference between the treatment groups in change from baseline) on cAKUSSI was seen. The cAKUSSI consists of clinically meaningful outcomes such as fractures, ruptures and joint replacements among others. The adjusted mean increase in scores was 15·1 in control patients, and 6·7 in the nitisinone group over the duration of the study, a reduction of nearly 56%, equivalent to a difference of two joint replacements or one fracture or rupture, if the difference occurred only in a single feature rather than all the features as seen in the cAKUSSI in SONIA 2. There was a strong trend toward fewer ruptures in the nitisinone group than in the control, consistent with the decrease in observed ochronosis scores. Also, there was a trend towards fewer fractures in the nitisinone group than in the control, in keeping with the statistically significant difference in change from baseline, in BMD, between the treatment groups, in favour of nitisinone. In bisphosphonate fracture prevention studies, the increase in BMD is around 5% [23]; in SONIA 2, at 48 months the T-

scores descreased by 11.9% in the control group, and apparently increased by 6.1% in the nitisinone group. Previous investigations have shown that stable or increased bone mineral density after bone-strengthening therapy is associated with fracture-protection.[24,25]

Amelioration of pain is a crucial and constant requirement in patients with AKU. In this regard, the significant decrease in pain from baseline, both in joints and spine, in nitisinone-treated patients is important. The difference in change from baseline at Month 48 between treatment groups was, however, only statistically significant for the spine but showed a positive trend also for joint pain. The difference in pain between the control and treatment groups could explain the beneficial difference in SF36 and active range of motion between the two groups.

There were more AEs reported in the nitisinone group than in the control group partly due to more reports of infections and infestations, eye disorders, and weight gain. There is no obvious explanation for the higher number of infections and infestations and no known mechanism by which nitisinone could increase infections. This has not been observed in the previous experience with nitisinone in HT-1. Tyrosine-related eye disorders were not unexpected, considering that the patients were not actively managed on a truly low-protein diet, and that nitisinone-treated patients had s-Tyr concentrations well above 500 µmol/L. Due to study logistics serum tyrosine was not measured at the time of keratopathy. Serum tyrosine was only measured during study site visits. In fact, the majority of the nitisinone-treated patients (86%) did not develop tyrosine-related symptoms despite very high serum tyrosine. Also, all patients who developed keratopathies did so during the first three years of the study. During year four there were no new cases.

In patients with keratopathies, lowering the nitisinone dose to 2 mg/day resulted in only minor decreases in s-Tyr, in agreement with results from previously reported dose-response study, and recurrent keratopathies were seen in several of those patients.[26] No direct relationship between tyrosine levels and occurrence of these events could be seen. It is likely that it is the ocular tyrosine concentrations that are key to causing keratopathy rather than those in the serum.

All patients were asked to reduce their protein intake. Decreasing dietary protein could have led to consumption of a diet containing more carbohydrates and fat, and this may be the explanation for the weight gain seen in the nitisinone group, who were probably more likely to make the dietary change, as they were made aware of the risk of developing tyrosine-related ocular symptoms. In patients who develop keratopathies, plasma tyrosine levels should be

monitored. A diet restricted in tyrosine and phenylalanine should be implemented to keep the plasma tyrosine level below 500 µmol/L. In addition, nitisinone should be temporarily discontinued and reintroduced only when the keratopathy has resolved.

There were some limitations in SONIA 2. The inability to blind patients to nitisinone led to a trial design that may have affected the results of some subjective variables, including possibly leading to an under-reporting of AEs in the control group. Morbid events such as fractures and ruptures were studied in an unselected population. For example, fracture intervention trials traditionally have been carried out in homogenous populations all having osteoporosis at recruitment; in SONIA 2 only a proportion had osteopenia at recruitment, affecting the statistical significance of outcomes such as fractures and others such as ruptures [27]. The age of patients varied from around 25 to over 70 years, with a large variation in disease severity. There were more dropouts than anticipated and this was due to disabled and immobile patients having to travel long distances to attend the study. It was especially hard to motivate the control patients to attend the final visit at Month 48. Further, it was not possible to put in place dietetic management of the expected tyrosinaemia due to patients being dispersed all over Europe and Jordan; such a measure could have possibly reduced drop-outs due to keratopathies in the nitisinone group. In addition, a longer trial could have provided further insights for this slowly progressive disease, however this was logistically not feasible.

In conclusion, we have shown that nitisinone 10 mg daily offers a 'biochemical cure' of AKU, demonstrated by the marked decreases in urine and serum HGA. For the first time a randomised research study has shown that nitisinone also reverses the ochronotic process shown by reduction in ear pigment, and reduces the rate of disease progression revealed by a lower cAKUSSI score in the nitisinone group.

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Author contributions

LRR, JAG, NS – pioneered the idea for SONIA 2, secured funding, and managed the study, drafting manuscript and final approval of the manuscript

AKH – finalising SONIA 2 logistics, writing protocol, serving as a medical monitor, drafting manuscript and final approval of the manuscript

AMM, ATH, ASD, ES, BPN, JHH – carried out the metabolic analyses, drafting manuscript and final approval of the manuscript

FG, DB, AS, AZ – carried out the biomarkers and genetic analyses, drafting manuscript and final approval of the manuscript

MK, HB, EL, RF, MF, MB, EW, CW, SV, AM, ST, NB – At RLUH in Liverpool assisted in conduct of study, as well as in advising on the various investigations, assessments and processes used in SONIA 2, drafting manuscript and final approval of the manuscript

JBA, KHLQS – Assisted with conduct of study in Paris, drafting manuscript and final approval of the manuscript

HG, RS, RI, VM, OL, EZ, EV, JS, JR - Assisted with conduct of study in Piešťany, drafting manuscript and final approval of the manuscript

JPD – At University of Liverpool assisted in conduct of study, drafting manuscript and final approval of the manuscript

EEP, TFC – planning and carrying out all statistic aspects of the study, drafting manuscript and final approval of the manuscript; TFC contributed to study design; EEP took over as the Main Trial Statistician after TFC retired

CvK, DL – management and coordination of clinical trial, drafting manuscript and final approval of the manuscript

BO, MR, JSM - contributed to study design and interpretation of the results, drafting the manuscript and final approval of the version to be published

AB - contributed to interpretation of the results, drafting the manuscript and final approval of the version to be published

JCJ, NPR – editing manuscript, as well as planning and securing funding for the study, drafting manuscript and final approval of the manuscript

CS – study conduct and drafting manuscript and final approval of the manuscript

Declaration of interests

BO/JSM/AB/MR reports personal fees and other from Swedish Orphan Biovitrum during the conduct of the study.

CS/NS disclosed that the AKU Society received £10,000 grant towards organising an AKU Patient Workshop

LR reports grants from the European Commission during the conduct of the study

FG reports grant from the EU during the conduct of the study, other from Nordic Bioscience, outside the submitted work.

AKH reports grants from the EU (the FP7 grant), other from Cudos B.V., other from PSR Group B.V., during the conduct of the study.

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Data-sharing statement for SONIA 2

Data access will be granted in response to qualified research requests. All de-identified individual participant data, for patients with separate consent signed for this purpose, can be made available to researchers. Data will be shared based on: the scientific merit of the proposal – i.e. the proposal should be scientifically sound, ethical, and have the potential to contribute to the advancement of public health as well as the feasibility of the research proposal – i.e. the requesting research team must be scientifically qualified and have the resources to conduct the proposed project. The data files would exclude data dictionaries that require user licenses. Data could be made available following finalized regulatory authority review and end of any data exclusivity periods and ending after 36 months or until corresponding author is able to fulfil this obligation whichever is earlier. Further, the study protocol and statistical analysis plan can be made available. Proposals should be directed to j.a.gallagher@liverpool.ac.uk to gain access. Data requestors will need to sign a data access agreement.

Legend to Tables

Table 1. Demographic data and baseline characteristics (FAS)

Table 2. HGA and other continuous efficacy variables in AKUSSI (FAS)

Table 3. Overall summary of adverse events (Safety analysis set)

Legend to figures

Figure 1. SONIA 2 (CONSORT) Flow Diagram

Figure 2. (a) u-HGA 24 (µmol)and (b) s-HGA over time (FAS)

Figure 3. (a) cAKUSSI and (b) mAKUSSI scores over time (FAS)

Suitability of Nitisinone in Alkaptonuria 2 (SONIA 2) - An international, multicentre, randomised, evaluator-blinded, no-treatment controlled, parallel-group study to assess the efficacy and safety of once daily nitisinone in patients with alkaptonuria after 12 months of treatment, followed by an additional 36-month treatment period

Ranganath LR[1,8], Psarelli EE[2], Arnoux JB[3], Braconi D[4], Briggs M[5], Bröijersen A[6], ~~Burgess~~ Loftus N[7], Bygott H[1], Cox TF[2], Davison AS[1], Dillon JP[8], Fisher M[9], Fitzgerald R[10], Genovese F[11], Glasova H[12,13], Hall AK[14], Hughes AT[1], Hughes JH[8], Imrich R[12,15], Jarvis JC[16], Khedr M[1], Laan D[17], Le Quan-Sang KH[3], Luangrath E[1], Lukáčová O[15], Milan AM[1], Mistry A[18], Mlynáriková V[15], Norman BP[8], Olsson B[6], Rhodes NP[8], Rovensky J[15], Rudebeck M[6], Santucci A[4], Shweihdi E[1], Scott C[19], Sedláková J[15], Sireau N[19], Stančík R[15], Szamosi J[6], Taylor S[7], van Kan C[17], Vinjamuri S[20], Vrtíková E[15], Webb C[21], West E[22], Záňová E[15], Zaťková A[12], Gallagher JA[8]

[1]Departments of Clinical Biochemistry and Metabolic Medicine, Liverpool University Hospitals NHS Foundation Trust (LUH), Prescot Street, Liverpool, L7 8XP, UK; [2] Liverpool Clinical Trials Centre, University of Liverpool, Block C, Waterhouse Building, Liverpool L69 3GL, UK; [3]Hôpital Necker-Enfants Malades, Paris Cedex 15, France; [4]Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Siena, Italy; [5]Department of Ophthalmology, LUH; [6]Swedish Orphan Biovitrum AB (publ), Stockholm, Sweden; [7]Department of Physiotherapy, LUH; [8]William Henry Duncan Building, West Derby Street, Liverpool, L7 8TX; [9]Department of Cardiology, LUH; [10]Department of Clinical Pharmacology, LUH; [11]Nordic Bioscience, Herlev, Denmark; [12]Institute of Clinical and Translational Research, Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovakia; [13]Institute of Pharmacology and Clinical Pharmacology, Slovak Medical University, Bratislava, Slovakia; [14]Cudos BV, Hoofddorp, Netherlands; [15]National Institute of Rheumatic Diseases, Piešťany, Slovakia; [16]School of Sport and Exercise Science, Liverpool John Moores University, Liverpool, UK; [17]PSR group B.V., Hoofddorp, Netherlands; [18]Department of Radiology, LUH; [19]AKU Society, Cambridge, UK; [20]Department of Nuclear Medicine, LUH; [21]Department of ENT, LUH; [22]Department of Dermatology, LUH.

Corresponding author: LR Ranganath, Department of Clinical Biochemistry and Metabolic Medicine, Royal Liverpool University Hospital, Prescot Street, Liverpool, L7 8XP; e-mail: lrang@liv.ac.uk

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Supplementary references - 6

Keywords: alkaptonuria, nitisinone, homogentisic acid, randomi~~z~~sed clinical trial, outcome, safety, ochronosis, AKUSSI

Abstract

Alkaptonuria (AKU) is a genetic, rare, multisystem disease, characterised by accumulation of homogentisic acid (HGA). There is no approved HGA-lowering therapy. Nitisinone decreases HGA generation. SONIA 2 investigated the effect of nitisinone on the disease process, and on progression of AKU.

Methods: This was a 48-month randomised, open-label, evaluator-blinded, parallel-group study performed in the UK, France and Slovakia. Patients ≥25 years of age with confirmed AKU and any clinical disease manifestations were randomisszed to receive either 10 mg nitisinone or no -treatment. Site visits were performed at 3 months and yearly thereafter. Results from history, photographs of eyes/ears, whole body scintigraphy, echocardiography, and abdomen/pelvis ultrasonography, were combined to derive the Alkaptonuria Severity Score Index (cAKUSSI). The primary objective was to show decrease in daily urinary HGA (u-HGA_{24}) excretion at month 12. Secondary objectives included ~~investigating~~ comparing clinical ~~benefit~~ outcomes after 48 months. ~~Nitisinone and control groups were compared.~~

Findings: 69 patients were randomised to nitisinone and 69 to the control group. 55 patients in the nitisinone group and 53 in the control group completed the study. u-HGA_{24} at 12 months was statistically significantly decreased by 99.7% in the nitisinone group compared with control. The adjusted geometric mean (ratio nitisinone/control) was 0·003 (0·003 – 0·004), ~~(~~p<0·0001~~)~~. ~~Ochronosis (pigmentation) of eyes (p=0·0011) and ears (p=0·02) decreased in the nitisinone group at 48 months but not in control.~~ cAKUSSI scores at 48 months decreased statistically significantly in nitisinone group compared with control [adjusted mean difference -8·6 (-16·0 – 1·2), ~~(~~p=0·02~~)~~]. The incidence of adverse events (AEs) was similar for the groups, but numerically more AEs were reported in the nitisinone group (400 AEs in 85·5% of patients) versus control (284 AEs in 82·6% of patients).

Interpretation: Nitisinone 10 mg daily was well tolerated and effective to reduce urinary excretion of HGA. Nitisinone decreased ochronosis and improved clinical signs, indicating a slower disease progression.

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Research in context

Evidence before this study

There has been only one previous long-term clinical study using the potentially disease-modifying agent, nitisinone, to evaluate the effect of the drug on AKU disease progression (Medline search up to and including February 2020). The terms used in the Medline search were nitisinone, alkaptonuria, and outcomes. In addition, because AKU is a rare disease, personal contacts with researchers and clinicians in the field enable us to confidently state that there has been only one previous outcomes trial using nitisinone in AKU. The National Institutes of Health (NIH, USA) nitisinone outcomes study on 20 nitisinone-treated and 20 control AKU patients, employed an improvement in the lateral rotation of the hip as the endpoint to decide on efficacy of 2 mg nitisinone daily over 3 years, the effect on this endpoint deemed inconclusive. There have been three short-term studies, two in the NIH, USA, and one in Liverpool, UK, that reported the metabolic efficacy of nitisinone in terms of lowering HGA. An audit of the use of nitisinone 2mg daily off-label in the National AKU Centre in Liverpool, funded by NHS England Highly Specialized Services, showed metabolic benefit, arrest of ochronosis, and slower progression of AKU disease, but this was an audit of a service rather than a research study.

Added value of this study

The present international, multicentre, randomi~~s~~sz~~e~~ed, controlled, evaluator-blinded, parallel-group study is an analysis of the efficacy of using 10 mg nitisinone daily in AKU. Both the nitisinone-treated and control groups had similar numbers of patients. The outcome was based on the effect of nitisinone on the change in AKU severity score index, a composite disease score, over four years. The power of the study was much increased by the use of the composite score, AKU severity score index. The composite disease score outcome was also clear cut in showing that nitisinone decreased the progression of AKU for the very first time in a randomi~~s~~sz~~e~~ed study. The study of the control group over four years has improved our understanding of the natural history further.

Implications of all the available evidence

We believe that the publishing of our manuscript will provide a major boost to the study of, and progress in, rare diseases. Our manuscript will be of interest to the general readership of the journal because AKU is an iconic disease whose study foreshadowed genomic medicine. AE Garrod applied Mendel's Laws of inheritance to human disease as early as 1902 in his studies of AKU. AKU has the added attribute of being a rare disease, in which the natural history is incompletely understood (an attribute it shares with most rare diseases); rare disease is 'common' in the sense that virtually all readers will need to manage rare diseases, with lessons to learn from our experience. The lack of patient numbers in which to study and carry out fully powered clinical trials, as in major frontline diseases like cardiovascular disease and diabetes, challenges the rare disease community to develop innovative ways to develop much needed therapies. We have employed a weighted composite score, which enabled us to overcome the low patient numbers by increasing power, and showing for the first time that nitisinone has disease-modifying attributes as well as metabolic efficacy. Our experience also

provides a powerful example of the effective re-purposing of an existing drug for a novel indication, which may be a more practical strategy than developing entirely new drugs for rare diseases. Our experience may empower researchers into the other 7000 rare diseases in their approach to achieve solutions in their diseases of interest. Finally, our findings demonstrate for the first time the efficacy of a disease modifying drug in the iconic disease AKU, and therefore bring hope to patients with this condition.

Background

Alkaptonuria (AKU) (OMIM 203500) is a rare, serious, autosomal recessive multisystem disorder[1] affecting approximately one in every 250 000 to 1 million people.[2] The disease was the first ever described in a paper by AE Garrod in 1902[3], in which Mendel's laws of inheritance were applied in human disease. Still, AKU lacks a pharmacological treatment. Genetic deficiency of homogentisate dioxygenase activity (HGD) results in accumulation of homogentisic acid (HGA) (Figure S1). HGA is then progressively deposited as yellow/dark pigment in connective tissue, rendering these more rigid and eventually brittle, and prone to degradation, a process termed ochronosis.[4,5] As the causal agent, HGA may therefore represent a suitable surrogate for a clinically meaningful endpoint in clinical trials. This was also suggested by the European Medicines Agency (EMA), during scientific advice before starting our clinical program. Degradation of ochronotic tissue is mainly responsible for the multisystem involvement, with varying phenotype, characterised by severe premature spondyloarthritis, lithiasis, cardiac valve disease, fractures, muscle and tendon ruptures, and osteopenia.[6,7] Palliative analgesia and arthroplasty is the mainstay of AKU therapy.

AKU is a disorder of tyrosine metabolism like another inherited condition known as hereditary tyrosinaemia type 1 (HT-1) (OMIM 276700). In HT-1, there is a deficiency of fumarylacetoacetate hydrolase, resulting in early liver and kidney disease and death in childhood if untreated.[8,9] Nitisinone (2-[2-nitro-4-(trifluoromethyl) benzoyl] cyclohexane-1,3-dione) is an inhibitor of the hydroxyphenylpyruvate dioxygenase (HPPD) (EC 1.13.11.27) and has been used in HT-1 since 1991. As activity of HPPD leads to formation of HGA (Figure S1) nitisinone was hypothesiszed in the late 1990s to be a potential treatment for AKU.[10] Following initial research of nitisinone for treatment of AKU[1,11], a three-year clinical trial comparing a nitisinone-treated patient group, receiving a 2 mg daily dose, with an untreated group, with 20 AKU patients in each group, was reported as inconclusive, despite showing excellent biochemical efficacy.[12]

Despite this setback, research into the use of nitisinone in AKU has continued. In addition, nitisinone 2 mg daily has been reimbursed for use in the United Kingdom's National Alkaptonuria Centre (NAC) since 2012, and a recent publication described positive outcomes for nitisinone in its metabolic and non-metabolic effects.[13,14,15] However, the off-label use in the NAC is, despite collecting high-quality data in a protocolised manner, in a service capacity and not a controlled clinical trial.

In designing the SONIA 2 study, it was assumed that the NIH trial did not succeed because of the small number of patients recruited, insufficient duration in such a very slowly progressive condition as AKU, the incomplete understanding of the natural history, and use of a single and possibly unreliable outcome measure in this multifaceted disease. An identification campaign to maximise patient recruitment for a new trial was subsequently carried out both in the UK and the rest of Europe.[15,16] A better understanding of the natural history and it's modification by nitisinone was shown in a mouse AKU model.[16,17,18,19] Careful phenotyping of the disease in a cohort of untreated AKU patients resulted in a composite score, termed AKU Severity Score Index (AKUSSI), a key factor when researching a multifaceted condition with variable phenotype.[19,20,20,21] In addition, a new clinical trial of nitisinone, with a considerably higher number of patients and a longer duration, was considered because of the naturally slow progression. The dose used in the inconclusive NIH trial was based on the experience of administering nitisinone to two AKU patients[10]; further, the EMA suggested finding a dose that normalises HGA, and therefore the issue of optimal dose was revisited in a dose-response study, the 'Suitability Of Nitisinone In Alkaptonuria 1' (SONIA 1).[21,22] In that study, the 8mg daily dose of nitisinone resulted in a mean reduction of u-HGA_{24} of 98.8 %, with a clear dose-response and much less variability compared with the other doses studied (1, 2 and 4 mg). An increase in tyrosine levels was seen at all doses but the dose-response relationship was less clear, with no tyrosine-related adverse events seen at any dose. Since the 8mg dose resulted in u-HGA_{24} close to normal values, a dose of 10 mg daily, which was achieved with an available capsule strength, was selected for the new trial, SONIA 2. All of these factors influenced the design of ~~the new trial called~~ SONIA 2~~.~~ in which ~~SONIA 2~~ the safety ~~(harm if any)~~ and efficacy of nitisinone 10 mg daily in AKU was investigated.

METHODS (additional details available in supplementary material)

Objectives

The primary objective in SONIA 2 was to demonstrate that nitisinone was superior compared to control in reducing u-HGA_{24} in patients with AKU after 12 months. Secondary objectives were defined to demonstrate the sustained control of urinary and serum HGA up to 48 months and to demonstrate the effect on clinical parameters and to assesses the safety of nitisinone in AKU.

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Study design

SONIA 2 was a four-year, open-label, evaluator-blinded, multicentre, randomis~~z~~ed, no-treatment controlled, parallel-group study. A formal interim analysis was planned when all patients completed 12 months of treatment. This analysis included the complete set of efficacy and safety data up to 12 months, thus including the final analysis of the primary endpoint. The purpose was to ~~see if it was possible to submit an~~evaluate if data demonstrated results suitable for a regulatory application ~~for the new indication to the EMA~~ already at that stage, even though the study was to continue for another 3 years to collect more complete efficacy and safety data. The study design is summaris~~z~~ed in Figure S2~~1~~.

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The study was performed at three investigational sites: Liverpool (UK), Paris (France) and Piešťany (Slovakia). Independent Ethics Committee at each centre approved the study.

Patients

The aim was to recruit 140 patients aged 25 years or older, with a confirmed diagnosis of AKU and ~~some~~any clinical ~~symptoms~~manifestation in addition to increased HGA; 70 randomis~~z~~ed to nitisinone 10 mg and 70 to a control (no -nitisinone) group. ~~Patients were required to have a clinical manifestation of AKU.~~ All patients provided written informed consent prior to inclusion.

Treatment

Oral nitisinone (Orfadin®) 10 mg daily was administered in the treated group. The control group dd not receive nitisinone.

Nitisinone was withdrawn in patients who developed signs of ocular tyrosine-related adverse events (AEs). If feasible, once the symptoms had resolved (minimum 2 months after temporary withdrawal), nitisinone was reintroduced at a lower dose (2 mg daily). Alternatively, the patient was withdrawn from the study. If ocular tyrosine-related symptoms reappeared on the lower dose, nitisinone was permanently withdrawn and the patient was monitored until the symptoms resolved.

There were no restrictions regarding concomitant medications. Patients in both groups could freely use e.g. analgesics, anti-inflammatory drugs and others as needed to treat symptoms of AKU.

Randomisation and masking

Patients were randomly assigned to one of the two groups in a 1:1 ratio. The randomisation was stratified by study centre and age (≤ 55 years and > 55 years) and was carried out by using randomly permuted blocks (4 patients/block) within each study centre and age stratum. The study statistician created a program to randomly assign the patients to the two treatment groups using the SAS System. The randomisation was centrally implemented in the electronic CRF system (Viedoc®).

It is not possible to ~~adequately~~ blind a study with nitisinone in AKU because one of the signs of the disease is that the urine darkens due to oxidation of excreted HGA. Patients can therefore easily notice if they are receiving active drug or not. Therefore, the control group received no placebo treatment. Instead, the study was evaluator-blinded as far as possible. Assessments which did not require direct contact between the evaluator and the patient (such as evaluation of images) were blinded during the entire study. The blinded evaluators were experts in their respective field, and never met the patients. Other assessments were made by objective measurements, such as that of bone density. ~~The only subjective reporting in the study was that of adverse events and pain.~~It is, however, recognised that reporting of subjective assessments may have introduced bias for some of the secondary endpoints, such as pain, quality of life assessments, and reporting of adverse events.

Procedures

In addition to a 24-h urine (u-HGA_{24}) collected into acid for HGA and creatinine determination, fasting acidified serum for HGA, tyrosine and creatinine, a number of assessments and investigations were carried out (supplementary material). These included collection of medical history and physical examination, including those specific for AKU, a wide range of clinical outcome measures, including range of motion tests and quality of life assessments, safety assessment and other procedures shown in Table S1 and elsewhere.[13~~4~~,14~~5~~]

AKU Severity Score Index (AKUSSI) assessments (Table S1)

The AKUSSI incorporates multiple, clinically meaningful AKU outcomes that can be described in a single score.[13~~4~~,19~~20~~,20~~1~~] All items included in the AKUSSI were assessed at baseline and yearly thereafter.~~-~~ Two types of AKUSSI were included as secondary outcomes in SONIA 2. These are the clinical evaluation AKUSSI (cAKUSSI) and a modified AKUSSI (mAKUSSI = cAKUSSI without pigmentation features).

Patients visited study sites at 3 months, and then annually up to month 48; a close-out phone call took place at month 49. A questionnaire, completed by patients, collected safety information at 6, 18, 30 and 42 months.

~~**Outcomes**~~

~~The primary objective in SONIA 2 was to demonstrate that nitisinone was superior compared to control in reducing u-HGA$_{24}$ in patients with AKU after 12 months. Secondary objectives were defined to demonstrate the sustained control of urinary and serum HGA up to 48 months and to demonstrate the effect on clinical parameters.~~

At each visit, AEs and laboratory values were recorded. AEs included clinically significant signs and symptoms and abnormal test findings (e.g. laboratory analysis results, vital signs or ECG) that the investigator considered clinically significant and/or that led to a medical/surgical intervention including withdrawal of nitisinone or discontinuation from the study.

Statistical analysis

Only a few subjects would be needed to detect a statistically significant effect on the primary endpoint, u-HGA$_{24}$. Therefore, the sample size was based on the AKUSSI score, to allow the possibility to establish an effect on a clinical endpoint. ~~Despite u-HGA$_{24}$ being the primary efficacy endpoint, the sample size estimation was based on clinical outcome, i.e.,~~Using data from a cross-sectional study of AKU using AKUSSI[13,14,15,20] and follow-up data~~.~~ ~~I~~it was assumed that if nitisinone reduce~~d~~s the mean increase in AKUSSI over the 4-year period to 4 points, compared to 8 points in the control group, and taking the standard deviation of the increase to be 8, then a sample size of 64 per group was required for a two-sided t-test with 80% power for a significance level 0·05. With an estimated 10% drop-out rate, a sample size of 70 per group was required (140 patients in all).

The Full Analysis Set (FAS) including all randomi~~z~~sed patients was used for the analysis of efficacy variables, containing all randomi~~z~~sed patients who had a valid u-HGA$_{24}$ at baseline. The Safety Analysis Set was used for the analysis of safety variables. All randomi~~z~~sed patients were included in both sets.

All statistical analyses were performed with the SAS System (version 9.3~~ or later~~, SAS Institute, Cary, NC). Two-sided 95% confidence intervals corresponding to a two-sided 5% level of significance were used throughout the analyses. All relevant study data were tabulated with descriptive statistics, including mean, standard deviation, standard error of the mean,

median, minimum and maximum for the continuous variables, and frequencies and proportions for the categorical variables. Both absolute values and changes from baseline were tabulated, if feasible. No allowance for multiplicity was made.

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Analysis of primary endpoint

The primary endpoint was the u-HGA_{24} in patients with AKU after 12 months.

A longitudinal model (mixed model for repeated measures (MMRM)) with an underlying normal distribution was fitted for the analysis of the primary endpoint. An unstructured covariance matrix was used along with a restricted maximum likelihood method (REML), while the degrees of freedom were estimated using Kenwood-Rogers method. Treatment, site, age category, visit and treatment by visit interaction were added as fixed factors in the model together with the baseline log(u-HGA_{24}) value as a covariate and with subject-within-site included as a random factor. The analysis was performed using the log(u-HGA_{24}) as dependent variable. Model based point estimates and associated two-sided 95% confidence intervals were calculated.

Analysis of secondary endpoints supporting primary endpoint

u-HGA_{24} at month 3, 24, 36 and 48 was analysed using the same MMRM model as in the primary endpoint analysis.

Analyses of other endpoints

Changes from baseline in cAKUSSI, mAKUSSI, individual AKUSSI items, and pre-dose s-HGA~~s-HGA~~, were also analysed. For continuous secondary endpoints, the same statistical model as in the primary endpoint analysis was used, with the exception that these analyses were conducted on the original scale without transformation. However, this was not the case for s-HGA and s-Tyr where log transformation was used. Ordinal secondary endpoints were modelled using a generalised estimating equations (GEE) approach, whereas count data was modelled using an MMRM with an underlying Poisson distribution.

Analysis of safety data

All adverse events (AEs) during the study were coded using the Medical Dictionary for Regulatory Activities (MedDRAv.16.0). The incidence of AEs was summarised in frequency tables. The changes in safety laboratory parameters from baseline to all post-baseline visits were summarised by treatment group and visit using descriptive statistics. These included

serum concentration of clinical chemistry, haematology, vital signs, electrocardiogram (ECG) and corneal eye assessments.

A Data Monitoring Committee was assigned to safeguard the interests of study participants and to continuously monitor the safety of the patients in the study.

The study was registered at clinicaltrials.gov (NCT01916382).

Role of the Funding Source

This study was funded by a grant from the European Union Framework Programme 7 (DevelopAKUre, project number: 304985). The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

~~All metabolic and non-metabolic data presented and discussed in this manuscript is shown in Table 1.~~

Disposition of patients: 139 patients were screened and 138 ~~patients were recruited to~~included the study between 7th May 2014 and 16th February 2015, with 69 patients randomi~~s~~zed to each of the two study groups. SONIA 2 was funded by the European Commission under their FP 7 programme, with a strict time limit for its completion. Therefore, as the number of recruited patients was deemed sufficient at the end of the recruitment period, ~~we were forced to end~~ recruitment was ended after 138 patients were included (139 screened) to meet these timelines. Of these, 108 patients completed the study. The main reason for discontinuation in the control group was withdrawn consent (10 patients), while AEs were the most common reason for withdrawal (nine patients) in the nitisinone group (~~Table 2;~~ Figures ~~2~~ 1 and S2).

Demographic data and other baseline characteristics: The two groups were well balanced. The majority of the patients (134 patients, 97·1%) were Caucasian. There were more males in the nitisinone-treated group (45 patients, 65·2%) compared to the control group (40 patients,

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58·0%). The mean age was slightly lower in the control group compared to the nitisinone group (Table 1~~3~~).

Efficacy and~~,~~ safety~~, pharmacokinetic, and genetic~~ assessments

Primary outcome - Urinary HGA: The u-HGA_{24} was statistically significantly decreased in the nitisinone-treated group compared to the control at all visits after baseline. These findings were consistent irrespective of age, sex, or study site. At month 12, the time of evaluation of the primary endpoint, the adjusted mean u-HGA_{24} had statistically significantly decreased by 99·7% in the nitisinone group compared to the control group [adjusted geometric mean (ratio nitisinone/control) 0·003 (0·003 – 0·004), p<0·0001] (Table 2~~1~~, Figure 2~~3~~A).

Serum HGA: At baseline, the geometric mean s-HGA was comparable for the two study arms. At month 12, the adjusted geometric mean s-HGA in the nitisinone group had statistically significantly decreased by 98·8% compared to the control group [adjusted geometric mean (ratio nitisinone/control) 0·01 (0·01 – 0·02)]. At each visit after baseline, the difference in change from baseline in s-HGA between the study arms was statistically significant (p<0·0001~~p<0.0001~~) (Table 2~~1~~, Figure 2~~3~~B).

Interim analysis of secondary efficacy outcomes: The 12-month analysis of the secondary efficacy endpoints did not support an application to ~~the~~a regulatory authority ~~EMA~~ for the new indication.

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AKUSSI (cAKUSSI and mAKUSSI) assessments: At baseline, cAKUSSI was slightly higher in the nitisinone group than in the control group. Over time there was an increase in cAKUSSI in the control group from baseline to month 48, while there was less of an increase in the nitisinone group. The difference between the two groups in the change from baseline to month 48 was statistically significant [adjusted mean difference -8·6 (-16·0 – 1·2), (p=0.02)]. The adjusted mean increase was 15·1 and 6·7 points in the control and nitisinone groups respectively, over the duration of the study (Tables 2~~1~~ & S1, Figure 3A~~4~~).

mAKUSSI: At month 48 there was no ~~notable~~ statistically significant difference between the two groups in change from baseline [adjusted mean difference -3·6 (-9·6 – 2·4), p=0·23] (Tables 2~~1~~ & S1). There was, however, a continuous increase in mAKUSSI in the ~~untreated~~

control group from baseline to Month 48, while a slower increase was observed for the nitisinone group (Tables 2 & S1, Figure 3A).

Selected individual AKUSSI items

Statistically significant differences between the two treatment groups were observed at Month 48, and for some variables also from earlier time points, for the following variables.

- cAKUSSI.
- Eye pigmentation (Table 2, Figure S3A)
- Ear pigmentation (Table 2, Figure S3B)
- Osteopenia of the hip (T-scores for bone density) (Table 2, Figure S4A)
- Number of spinal regions with pain (Table 2, Figure S5B)
- Self-evaluated transition (in SF-36) (Figure S6)

For the number of joints with pain, a statistically significant difference in favour of nitisinone was observed at Month 12 [adjusted mean difference -0·9 (-1·6 – -0·1), p=0·022]. Numerically, the difference between the groups was relatively constant at subsequent visits, and at Month 48, [adjusted mean difference -0·7 (-1·6 – 0·1), p=0·10].

An increasing gap, between the two treatment groups from baseline to Month 48, supporting a lower rate of disease progression in the nitisinone group, was observed for the following variables, however the result failed to reach statistical significance (p-values>0·05):

- mAKUSSI (Table 2, Figure 3B)
- Number of fractures (Table 2, Figure S4B)
- Number of tendon, ligament and muscle ruptures (Table 2, Figure S4C)

Other key secondary outcomes

Consistent trends towards better outcome in the nitisinone group compared to untreated controls were also observed for:

- Range of motion of the joints (Figure S7)
- Self-evaluated transition (in SF-36) (Table S8)
- Quality of life (SF-36) (Figure S6)

No notable difference between the treatment groups was observed for any of the other variables.

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Eye pigmentation: At baseline, the mean pigmentation score was higher in the nitisinone group than in the control group. From month 12 to month 48 there was a steady increase in eye pigmentation scores in the control group while the scores remained at a fairly constant level in the nitisinone group. The difference between the two groups was significant at month 48 (p=0·0011) (Tables 1 & S1, Figure 5A).

Ear pigmentation: At baseline, the mean ear pigmentation score was comparable for the two treatment groups. There was a steady increase in ear pigmentation in the control group, while there was a decrease in the nitisinone group. The adjusted mean difference in the change from baseline was significant at all visits (Tables 1 & S1, Figure 5B).

Bone mineral density (BMD) of the hip: At baseline, there was a slightly higher number of patients with lower than normal BMD (T-scores < -1·0) in the nitisinone group compared to the control group. At month 48, there was a significant difference in favor of nitisinone between the treatment groups, in changes in T-scores from baseline (p=0·05) (Tables 1 & S1, Figure 6A).

Fractures: At baseline, the same number of adult fractures were reported for both treatment groups. At month 48, the number of patients who experienced fractures after baseline was numerically higher in the control group than in the nitisinone group. This difference was not statistically significant (p=0·16). There was, however, a trend towards an increase over time in the number of fractures in the control group compared to nitisinone-treated patients (Tables 1 & S1, Figure 6B).

Tendon, ligament and muscle ruptures: At baseline, more patients in the control group had experienced at least one rupture compared to patients in the nitisinone group. Over time, there was a steady increase in the control group in cumulative number of new ruptures since baseline, while from month 12 there was only one new rupture in the nitisinone group, but the difference was not significant (Tables 1 & S1, Figure 6C).

Joint pain: At baseline, the mean (SD) number of joints with pain was comparable in the two treatment groups, namely 4·6 (3·3) and 4·8 (3·0) joints, in the control and nitisinone groups respectively. At month 48, there was a decrease in the number of joints with pain in the nitisinone group; the adjusted mean [95% CI] change from baseline was -1·0 [-1·6; -0·3] joints. In the control group, the corresponding value was -0·2 [-0·9; 0·4]. The difference between the

~~two groups did not, however, reach significance although it should be noted that the observed difference was seen at all post-baseline visits (Tables 1 & S1, Figure 7A).~~

~~*Spine pain*: At baseline, the mean (SD) number of spinal regions with pain was comparable in the two treatment groups; 2·3 (1·2) and 2·3 (1·3) in the control and nitisinone groups respectively. There was a significant decrease in the number of spinal regions with pain in the nitisinone group at month 48 compared to baseline (p=0·05); the adjusted mean [95% CI] change from baseline was -0·6 [-0·9; -0·3] regions. In the control group, the corresponding value was -0·2 [-0·5; 0·2]. The difference between the two groups did not reach statistical significance (Tables 1 & S1, Figure 7B).~~

~~*Other results*: There were no differences seen for other AKUSSI features (Table 1).~~

Safety:

A total of 400 AEs in 85·~~.~~5% of patients in the nitisinone group and 284 AEs in 82·~~.~~6% of patients in the control group were reported. Most AEs reported were within the system organ class (SOC) "Musculoskeletal and connective tissue disorder" (mostly manifestations of AKU); 53 and 54 events were reported for 24 patients in the control group and 31 patients in the nitisinone group, respectively. "Infections and infestations" was the second most common SOC. There was a higher incidence of AEs in this SOC in the nitisinone group; 56 AEs were reported for 27 patients while in the control group there were 24 AEs reported for 11 patients. Pneumonia and bronchitis were more commonly reported in the nitisinone group than in the control group. Other than that, there was no clear pattern. "Eye disorders", the third most common SOC, were reported for 8 (11·6%) patients in the control group and 25 (36·2%) patients in the nitisinone group (Tables 3, S8, S9, S10, S11~~Tables 3, S6~~).

The incidence of AEs was 2·13 per patient years in the control group and 2·27 in the nitisinone group. The incidence of eye-related AEs was 0·3 and 0·96 respectively.

There were two deaths in the study, one due to heart failure and the other to myocardial infarction; both occurred in nitisinone-treated patients. None of the events was considered to be related to nitisinone treatment (Table ~~3~~???~~, S6~~).

A total of 53 patients, 26 in the control group and 27 in the nitisinone group, experienced at least one SAE during the study. None of these events was considered by the investigator to be related to nitisinone (Table S~~9~~4). The SOC "Musculoskeletal and connective tissue disorders"

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had the highest number of SAEs, most of them related to joint replacements, fractures and other manifestations of AKU.

Ocular adverse events: A total of 77 AEs in the SOC "Eye disorders" were reported. In the control group, 8 (11·6%) patients reported 12 events. In the nitisinone group, 25 patients (36·2%) reported 65 events. A majority of these, such as keratopathy (10 patients), eye pain (8 patients), dry eye (6 patients), increased lacrimation (4 patients), ocular hyperaemia (4 patients), eye irritation (3 patients), are considered related to the increased levels of tyrosine caused by nitisinone treatment (Tables S9, S10).

Nine of the patients in the nitisinone group developed tyrosine-related keratopathy in one or both eyes confirmed by slit-lamp examination. One further patient, who could not come for a follow-up visit, was withdrawn due to suspected keratopathy based on convincing ocular symptoms. Of the nine keratopathy patients confirmed by slit-lamp examination, eight had other eye symptoms, such as pain, blurred vision or other signs. One patient reported no symptoms before keratopathy was seen by slit-lamp at a pre-planned visit. In these nine patients with keratopathy, complete resolution was shown at a follow-up visit at least 2 months after nitisinone withdrawal. Eight patients restarted nitisinone at a dose of 2 mg/day after the recovery; five of those had recurrent symptoms while three were still asymptomatic at the end of the study (Tables 3, ~~& S9~~6).

As expected, serum tyrosine (s-Tyr) concentrations were above 500 µmol/L in all nitisinone-treated patients. At Month 12, the median value was 925 µmol/L, with a range from 563 to 1530. Decreasing the dose in those who switched from 10 to 2 mg following keratopathy had a limited effect on s-Tyr, with all patients still having levels above 500 µmol/L (Table S9, ~~Table 1,~~ Figure S8~~3C~~).

DISCUSSION

The direct cause of morbidity in AKU is HGA accumulation, resulting from genetic HGD deficiency.[2~~2~~4] HGA is therefore a surrogate for a clinically meaningful endpoint in clinical trials. In SONIA 2, u-HGA_{24} decreased markedly in the nitisinone-treated group compared to controls at all visits after baseline, and the primary objective of the study was thus met. Nitisinone efficiently decreased both u-HGA_{24} and s-HGA in nitisinone-treated patients, with mean values at month 12 decreasing by greater than 98% compared to control for both variables.

The difference between the groups in change in pigmentation, i.e. the ochronosis, which is the fundamental patho-physiological process in AKU, was statistically significant. This indicates that treatment with nitisinone arrested the ochronosis process in the eye and reversed it in the ear, by decreasing the accumulation of HGA. The crucial importance of ochronosis in AKU has recently been highlighted.[22,26] Reversal of the disease process ~~in AKU~~ in the ear was seen soon after starting nitisinone, and continued throughout the study duration. Although reversal of ochronosis in the ear was observed, the decrease in pigmentation was not total, and it is not clear whether a longer follow-up period would have shown more de-pigmentation.

A weighted composite score, the cAKUSSI, was used in SONIA 2, as for previously published data in AKU[13,34,13,35] This score was employed as it would have been 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 as employed in the NIH trial[12], given the ultra-rare nature of AKU, and the heterogeneous phenotypic severity. ~~At baseline I~~In SONIA 2, the baseline cAKUSSI scores were higher in the nitisinone group than in the controls. This may be because the nitisinone group was older, with an age difference in medians of three years, and containing more male patients, who have been shown to experience a more severe disease.[19,20,20,01]

In SONIA 2 a significant effect (difference between treatment groups ~~arms of the study~~in change from baseline) on cAKUSSI was seen. The cAKUSSI consists of clinically meaningful outcomes such as fractures, ruptures and joint replacements among others. The adjusted mean increase in scores was 15·1 in control patients, and 6·7 in the nitisinone group over the duration of the study, a reduction of nearly 56%, equivalent to a difference of two joint replacements or one fracture or rupture, if the difference occurred only in a single feature rather than all the features as seen in the cAKUSSI in SONIA 2. There was a strong trend toward fewer ruptures in the nitisinone group than in the control, consistent with the decrease in observed ochronosis scores. Also, there was a trend towards fewer fractures in the nitisinone group than in the control, in keeping with the statistically significant difference in change from baseline, in BMD, between the treatment groups, in favour of nitisinone. Previous investigations have shown that stable or increased bone mineral density after bone-strengthening therapy is associated with fracture-protection.[23,35,24,46]

Amelioration of pain is a crucial and constant requirement in patients with AKU. In this regard, the significant decrease in pain from baseline, both in joints and spine, in nitisinone-treated patients is important. The difference in change from baseline between treatment groups at

month 48 was, however, only statistically significant for the spine but showed a positive trend also for joint pain. The difference in pain between the control and treatment groups could explain the beneficial difference in SF36 and active range of motion between the two groups.

There were more AEs reported in the nitisinone group than in the control group partly due to more reports of infections and infestations, eye disorders, and weight gain. There is no obvious explanation for the higher number of infections and infestations and no known mechanism by which nitisinone could increase infections. This has not been observed in the previous experience with nitisinone in HT-1. Tyrosine-related eye disorders were not unexpected, considering that the patients were not actively managed on a truly low-protein diet, and that nitisinone-treated patients had s-Tyr concentrations well above 500 µmol/L. In fact, the majority of the nitisinone-treated patients (86%) did not develop tyrosine-related symptoms despite very high serum tyrosine. Also, all patients who developed keratopathies did so during the first three years of the study. During year four there were no new cases.

In patients with keratopathies, lowering the nitisinone dose to 2 mg/day resulted in only minor decreases in s-Tyr, in agreement with results from previously reported dose-response study, and recurrent keratopathies were seen in several of those patients.[2,5,7] No direct relationship between tyrosine levels and occurrence of these events could be seen. It is likely that it is the ocular tyrosine concentrations that are key to causing keratopathy rather than those in the serum.

All patients were asked to reduce their protein intake. Decreasing dietary protein could have led to consumption of a diet containing more carbohydrates and fat, and this may be the explanation for the weight gain seen in the nitisinone group, who were probably more likely to make the dietary change, as they were made aware of the risk of developing tyrosine-related ocular symptoms. In patients who develop keratopathies, plasma tyrosine levels should be monitored. A diet restricted in tyrosine and phenylalanine should be implemented to keep the plasma tyrosine level below 500 µmol/L. In addition, nitisinone should be temporarily discontinued and reintroduced only when the keratopathy has resolved.

There were some limitations in SONIA 2. The inability to blind patients to nitisinone led to a trial design that may have affected the results of some subjective variables, including possibly leading to an under-reporting of AEs in the control group. Morbid events such as fractures and ruptures were studied in an unselected population. For example, fracture intervention trials traditionally have been carried out in homogenously populations all having osteoporosis at

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recruitment; in SONIA 2 only a proportion had osteopenia at recruitment, affecting the statistical significance of outcomes such as fractures and others such as ruptures (~~2FIT6~~). The age of patients varied from around 25 to over 70 years, with a large variation in disease severity.~~; the lack of availability of recruitable particapants in such a rare disease was a key factor.~~ There were more dropouts than ~~we would have wished for and this was due to anticipated~~~~disabled and immobile patients having to travel long distances to attend the study~~. Further, i~~I~~t was not possible to put in place dietetic management of the expected tyrosinaemia with only once yearly visits, due to patients being dispersed all over Europe and Jordan~~, with no dietetic support already in place~~; ~~this~~ such a measure could possibly ~~could~~ have also reduced drop-outs due to keratopathies in the nitisinone group . In addition, a longer trial could have provided further insights for this slowly progressive disease, however this was ~~operationally difficult and~~logistically not feasible ~~to perform~~.

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In conclusion, we have shown that nitisinone 10 mg daily offers a 'biochemical cure' of AKU, demonstrated by the marked decreases in urine and serum HGA. For the first time a randomised research study has shown that nitisinone also reverses the ochronotic process shown by reduction in ear pigment, and reduces the rate of disease progression revealed by a lower cAKUSSI score in the nitisinone group.

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Author contributions

LRR, JAG, NS – pioneered the idea for SONIA 2, secured funding, and managed the study, drafting manuscript and final approval of the manuscript

AKH – finalising SONIA 2 logistics, writing protocol, serving as a medical monitor, drafting manuscript and final approval of the manuscript

AMM, ATH, ASD, ES, BPN, JHH – carried out the metabolic analyses, drafting manuscript and final approval of the manuscript

FG, DB, AS, AZ – carried out the biomarkers and genetic analyses, drafting manuscript and final approval of the manuscript

MK, HB, EL, RF, MF, MB, EW, CW, SV, AM, ST, NB – At RLUH in Liverpool assisted in conduct of study, as well as in advising on the various investigations, assessments and processes used in SONIA 2, drafting manuscript and final approval of the manuscript

JBA, KHLQS – Assisted with conduct of study in Paris, drafting manuscript and final approval of the manuscript

HG, RS, RI, VM, OL, EZ, EV, JS, JR - Assisted with conduct of study in Piešťany, drafting manuscript and final approval of the manuscript

JPD – At University of Liverpool assisted in conduct of study, drafting manuscript and final approval of the manuscript

EEP, TFC – planning and carrying out all statistic aspects of the study, drafting manuscript and final approval of the manuscript; TFC contributed to study design; EEP took over as the Main Trial Statistician after TFC retired

CvK, DL – management and coordination of clinical trial, drafting manuscript and final approval of the manuscript

BO, MR, JSM - contributed to study design and interpretation of the results, drafting the manuscript and final approval of the version to be published

AB - contributed to interpretation of the results, drafting the manuscript and final approval of the version to be published

JCJ, NPR – editing manuscript, as well as planning and securing funding for the study, drafting manuscript and final approval of the manuscript

CS – study conduct and drafting manuscript and final approval of the manuscript

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Declaration of interests

BO/JSM/AB/MR reports personal fees and other from Swedish Orphan Biovitrum during the conduct of the study.

CS/NS disclosed that the AKU Society received £10,000 grant towards organising an AKU Patient Workshop

LR reports grants from the European Commission during the conduct of the study

FG reports grant from the EU during the conduct of the study, other from Nordic Bioscience, outside the submitted work.

AKH reports grants from the EU (the FP7 grant), other from Cudos B.V., other from PSR Group B.V., during the conduct of the study.

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Data-sharing statement for SONIA 2

	SONIA 2 study data
Will individual participant data be available (including data dictionaries)?	Yes
What data in particular will be shared?	Individual participant data that underlie the results reported in this article, after de-identification (text, tables, figures, and appendices)
What other documents will be available?	Study protocol
When will data be available (start and end dates)?	Beginning 6 months and ending 36 months or until corresponding author is able to fulfil this obligation, whichever is earlier
With whom?	Researchers who provide a methodologically sound proposal as decided by a committee agreed within the consortium (Royal Liverpool Hospital, University of Liverpool, Alkaptonuria Society United Kingdom).
For what types of analyses?	To achieve aims in the approved proposal
By what mechanism will data be made available?	Proposals should be directed to lrang@liv.ac.uk; to gain access, data requestors will need to sign a data access agreement

Data access will be granted in response to qualified research requests. All de-identified individual participant data, for patients with separate consent signed for this purpose, can be made available to researchers. Data will be shared based on: the scientific merit of the proposal – i.e. the proposal should be scientifically sound, ethical, and have the potential to contribute to the advancement of public health as well as the feasibility of the research proposal – i.e. the requesting research team must be scientifically qualified and have the resources to conduct the proposed project. The data files would exclude data dictionaries that require user licenses. Data could be made available following finalized regulatory authority review and end of any data exclusivity periods and ending after 36 months or until corresponding author is able to fulfil this obligation whichever is earlier. Further, the study protocol and statistical analysis plan can be made available. Proposals should be directed to j.a.gallagher@liverpool.ac.uk to gain access. Data requestors will need to sign a data access agreement

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Legend to Tables

Table 1. Demographic data and baseline characteristics (FAS)

Table 2 HGA and other continuous efficacy variables in AKUSSI (FAS)

Table 3. Overall summary of adverse events (Safety analysis set)~~AEs in the most common SOCs, sorted by incidence rate in the nitisinone group (Safety analysis set)(merged with Table S7 (previous Table S6).~~

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Figure 1. SONIA 2 (CONSORT) Flow Diagram

Figure 2. (a) u-HGA 24 (µmol)and (b) s-HGA over time (FAS)~~Comparison of geometric mean u-HGA$_{24}$ (A), s-HGA (B) and s-Tyr (C) estimates between control and nitisinone treatment groups over 48 months in SONIA 2 (Error bars represent ±95% CI).~~

Figure 3. (a) cAKUSSI and (b) mAKUSSI scores over time (FAS)~~Comparison of mean cAKUSSI scores estimates at baseline and months 12, 24, 36 and 48 (Error bars represent ±95%CI).~~

~~Figure 4. Comparison of mean eye (A) and ear (B) ochronosis scores estimates at baseline and adjusted means at months 12, 24, 36 and 48 (Error bars represent ±95%CI).~~

~~Figure 5. Comparison of mean BMD estimates (T-scores) (A), fractures (cumulative numbers) (B) and ruptures (cumulative numbers) (C) at baseline and adjusted means at months 12, 24, 36, and 48 (Error bars represent ±95%CI).~~

Table . Demographic data and baseline characteristics (FAS)				
Variable	**Statistic**	**Control (n=69)**	**Nitisinone (n=69)**	**Total (n=138)**
Age (years)	n	69	69	138
	Mean (SD)	47·6 (10·1)	49·0 (11·3)	48·3 (10·7)
	Median (min; max)	48·0 (27; 67)	51·0 (26; 70)	49·0 (26; 70)
Body weight (kg)	n	69	69	138
	Mean (SD)	74·1 (15·6)	74·8 (14·8)	74·4 (15·1)
	Median (min; max)	73·0 (46; 122)	75·0 (36; 110)	74·0 (36; 122)
Height (cm)	n	69	69	138
	Mean (SD)	167 (9·5)	166 (9·2)	167 ()
	Median (min; max)	168 (148; 191)	168 (142; 189)	168 (142; 191)
Sex n (%)	Male	40 (58·0)	45 (65·2)	85 (61·6)
	Female	29 (42·0)	24 (34·8)	53 (38·4)
Race n (%)	White	67 (97·1)	67 (97·1)	134 (97·1)
	Black	0 (0·0)	1 (1·4)	1 (0·7)
	Asian	2 (2·9)	1 (1·4)	3 (2·2)

n: Number of patients observed.
Percentage calculated on n (patients in treatment groups).

Table 1. Demographic data and baseline characteristics (FAS)				
Variable	**Statistic**	**Control (n=69)**	**Nitisinone (n=69)**	**Total (n=138)**
Age (years)	Mean (SD)	47·6 (10·1)	49·0 (11·3)	48·3 (10·7)
Body weight (kg)	Mean (SD)	74·1 (15·6)	74·8 (14·8)	74·4 (15·1)
Height (cm)	Mean (SD)	167 (9·5)	166 (9·2)	167 (9·4)
Sex n (%)	Male	40 (58·0)	45 (65·2)	85 (61·6)
Race n (%)	White	67 (97·1)	67 (97·1)	134 (97·1)
	Black	0 (0·0)	1 (1·4)	1 (0·7)
	Asian	2 (2·9)	1 (1·4)	3 (2·2)
Centre n (%)	Liverpool	21 (30·4)	20 (29·0)	41 (29·7)
	Piešťany	32 (42·6)	33 (47·8)	65 (47·1)
	Paris	16 (23·2)	16 (23·2)	32 (23·2)

Table 2. HGA and continuous efficacy variables in AKUSSI

	Baseline	**Month 12**	**Month 48**
HGA			

HGA		Control	Nitisinone	Control	Nitisinone	Control	Nitisinone
u-HGA24 µmol	Mean (SD)	35394 (13869)	35019 (13124)	26444 (10397)	179 (398)	33207 (10160)	1569 (6220)
	Adjusted geometric mean (quotient nitisinone/control) with 95% CI	NA		0.003 (0·003 – 0·004)		0.005 (0·003 – 0·008)	
s-HGA mmol/L	Mean (SD)	28·26 (8·66)	30·35 (10·98)	28·93 (13·04)	0·71 (1·63)	37·08 (21·03)	2·80 (7·33)
	Adjusted geometric mean (quotient nitisinone/control) with 95% CI	NA		0.01 (0·01 – 0·02)		0.02 (0·02 – 0·03)	
AKUSSI							
cAKUSSI (points)	Mean (SD)	80·5 (33·4)	87·0 (34·2)	80·1 (34·7)	84·5 (33·7)	95·6 (36·0)	93·7 (37·8)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-2·5 (-5·7; 0·7)		-8·6 (-16·0; -1·2)	
mAKUSSI (points)	Mean (SD)	54·1 (24·9)	56·7 (26·7)	54·8 (25·7)	57·5 (26·8)	66·7 (29·7)	66·1 (31·1)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·5 (-2·5; 1·6)		-3·6 (-9·6; 2·4)	
Individual AKUSS items							
Eye ochronosis	Mean (SD)	14·1 (9·6)	17·3 (9·2)	14·7 (9·0)	16·8 (9·5)	16·4 (9·5)	16·5 (9·3)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·8 (-1·9; 0·3)		-2·5 (-3·9; -1·0)	
Ear ochronosis	Mean (SD)	3·9 (2·9)	4·1 (2·9)	4·0 (2·8)	4·1 (2·9)	4·0 (2·8)	4·0 (2·9)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·2 (-0·4; 0·0)		-0·5 (-0·9; -0·1)	
BMD (T-score)	Mean (SD)	-1·26 (0·98)	-1·3 (1·2)	-1·28 (0·98)	-1·39 (1·14)	-1·41 (0·81)	-1·22 (1·17)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·09 (-0·18; -0·01)		0·14 (0·00; 0·28)	
Aortic velocity (m/s)	Mean (SD)	1·6 (0·6)	1·8 (0·8)	1·6 (0·6)	1·8 (0·8)	1·7 (0·6)	1·8 (0·8)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·009 (-0·092; 0·075)		-0·030 (-0·149; 0·089)	
Joint pain	Mean (SD)	4·6 (3·3)	4·8 (3·0)	4·0 (3·1)	3·5 (2·7)	4·2 (3·3)	3·8 (2·7)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·9 (-1·6; -0·1)		-0·7 (-1·6; 0·1)	

Number of joints with osteoarticular disease	Mean (SD)	6·7 (3·2)	6·1 (3·1)	6·7 (3·2)	6·4 (3·2)	9·1 (3·3)	8·5 (3·6)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		0·0 (-0·1 ; 0·2)		-0·1 (-1·3; 1·1)	
Spinal pain	Mean (SD)	2·3 (1·2)	2·3 (1·3)	2·0 (1·2)	1·9 (1·3)	2·2 (1·4)	1·7 (1·3)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·2 (-0·5 ; 0·2)		-0·5 (-0·9; 0·0)	
Number of spinal regions with osteoarticular disease	Mean (SD)	3·0 (2·1)	3·4 (2·1)	3·0 (2·1)	3·4 (2·2)	3·5 (2·1)	3·7 (2·0)
		NA		0·0 (-0·3 ; 0·4)		-0·1 (-0·4; 0·3)	
Kyphosis (Cobb angles)	Mean (SD)	35·2 (10·2)	36·4 (10·6)	35·2 (9·2)	37·1 (10·7)	37·2 (7·8)	39·5 (9·7)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		0·9 (-0·3 ; 2·1)		1·0 (-0·8; 2·7)	
Scoliosis (Cobb angles)	Mean (SD)	10·5 (5·4)	10·8 (5·2)	10·5 (4·9)	10·7 (4·5)	11·8 (6·6)	12·1 (5·2)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		0·0 (-0·6 ; 0·6)		-0·1 (-1·6; 1·4)	

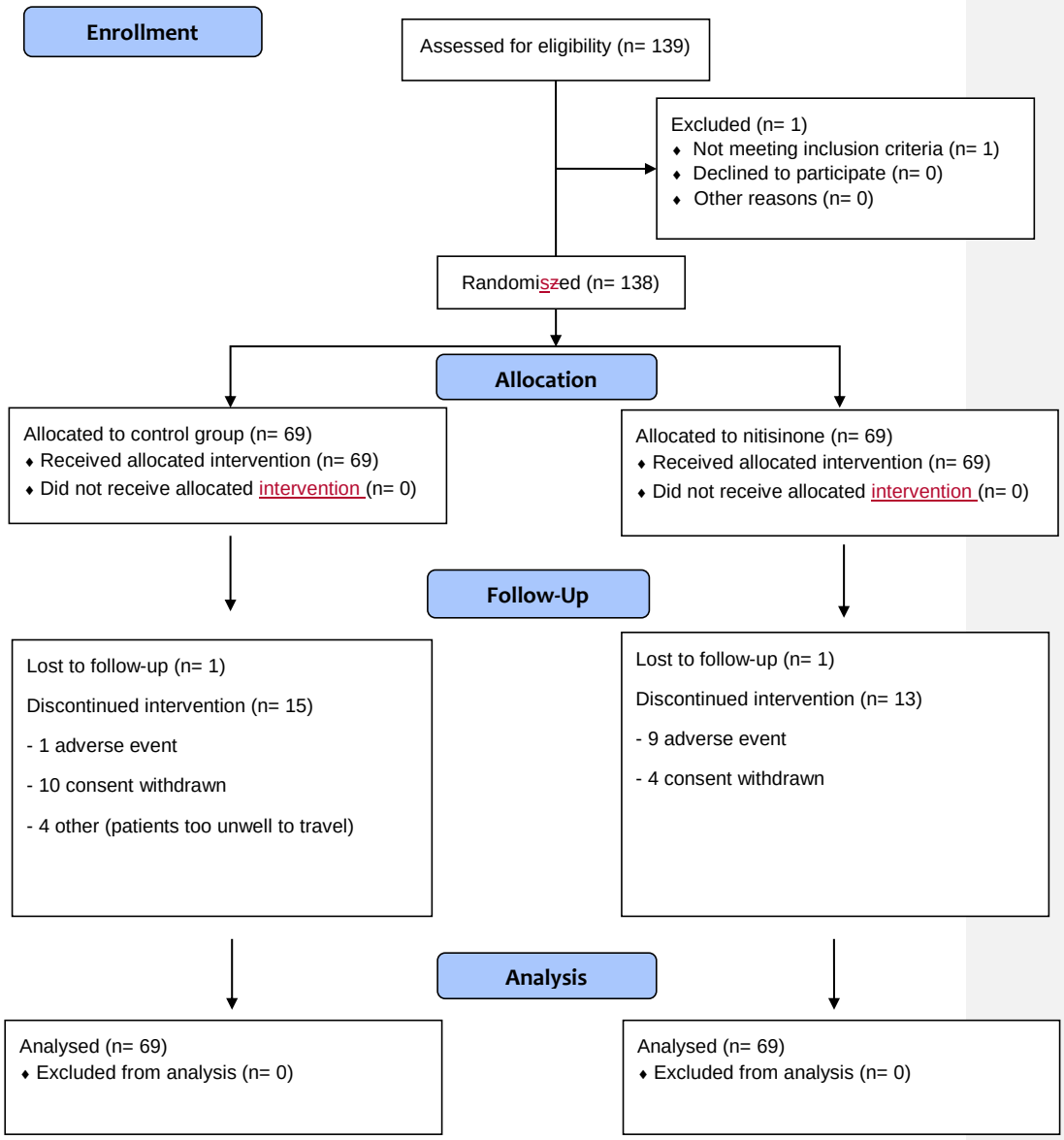
NA: Not applicable

Table 3.Safety

Table 3. AEs in the most common SOCs, sorted by incidence rate in the nitisinone group (Safety analysis set)				
	Control (n = 69, PYRS = 268)		**Nitisinone (n = 69, PYRS = 260)**	
SOC	**n (%) [E]**	**Incidence rate per 10 patient years**	**n (%) [E]**	**Incidence rate per 10 patient years**
Musculoskeletal and connective tissue disorders	24 (34·8) [53]	0·9	31 (44·9) [54]	1·2
Infections and infestations	11 (15·9) [24]	0·4	27 (39·1) [56]	1·0
Eye disorders	8 (11·6) [12]	0·3	25 (36·2) [65]	1·0
Investigations	10 (14·5) [12]	0·4	24 (34·8) [35]	0·9
Injury, poisoning and procedural complications	16 (23·2) [29]	0·6	19 (27·5) [28]	0·7
Skin and subcutaneous tissue disorders	9 (13·0) [10]	0·3	15 (21·7) [24]	0·6
Gastrointestinal disorders	13 (18·8) [17]	0·5	14 (20·3) [27]	0·5
Nervous system disorders	12 (17·4) [16]	0·4	14 (20·3) [26]	0·5
General disorders and administration site conditions	4 (5·8) [6]	0·1	10 (14·5) [10]	0·4
Vascular disorders	12 (17·4) [14]	0·4	8 (11·6) [10]	0·3
Cardiac disorders	10 (14·5) [11]	0·4	9 (13·0) [9]	0·3
Metabolism and nutrition disorders	13 (18·8) [19]	0·5	6 (8·7) [7]	0·2

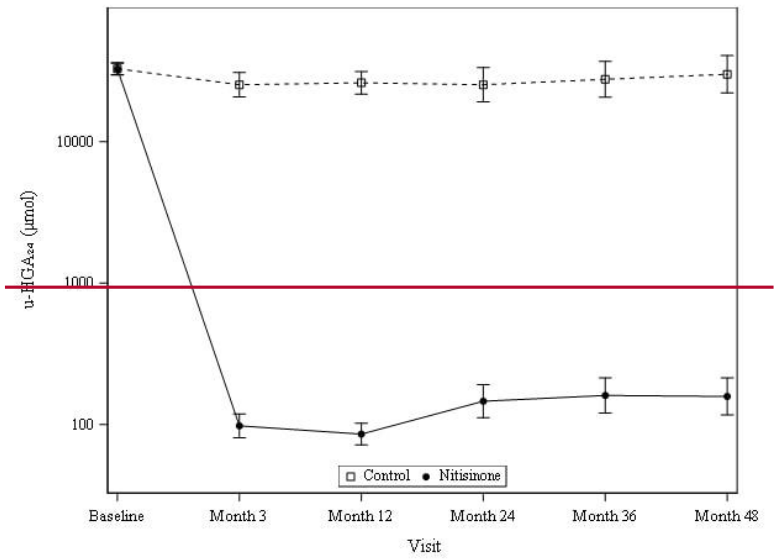
[a] Related to study drug, as judged by the investigator.
AE: Adverse Event n: Number of patients observed. NA: Not applicable SAE: Serious Adverse Event
Percentage calculated on n (patients in treatment groups).

~~Figure 1. SONIA 2 (CONSORT) Flow Diagram~~



~~Figure 2. Comparison of geometric mean u-HGA$_{24}$ (A), s-HGA (B) and s-Tyr (C) estimates between control and nitisinone treatment groups over 48 months in SONIA 2 (*p<0·05; **<0·01; ***<0·001) (Error bars represent ±95% CI).~~

~~Figure 2a- u-HGA 24 (µmol) over time (FAS)~~



~~Graph shows geometric mean (95% CI) for baseline and adjusted geometric mean (95% CI) for later time points; Y axis on log scale.~~

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~~Figure 2b – s-HGA (µmol/L) over time (FAS)~~

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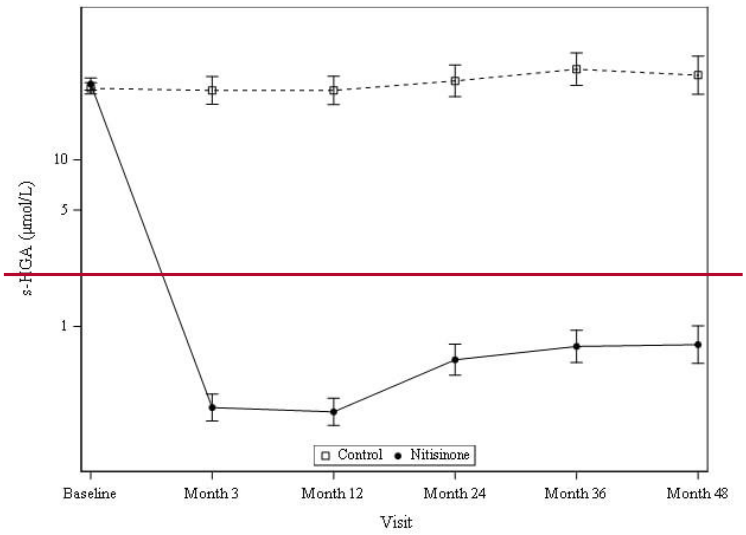

~~Graph shows geometric mean (95% CI) for baseline and adjusted geometric mean (95% CI) for later time points; Y axis on log scale.~~

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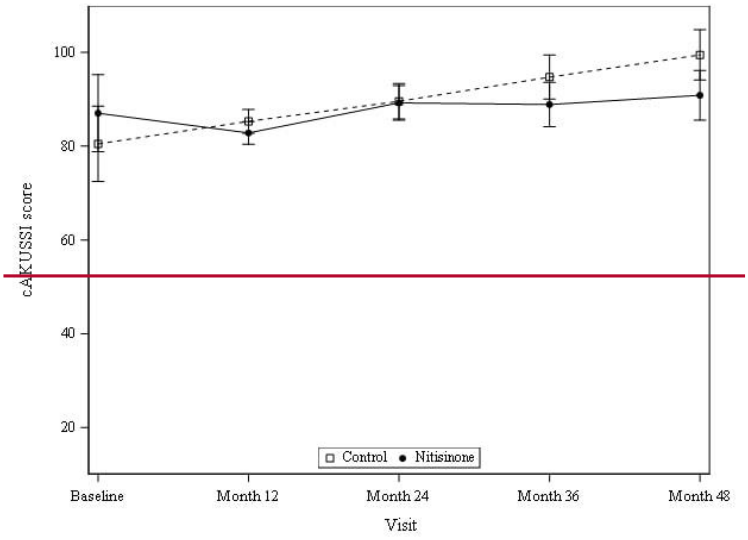
~~Figure 3. Comparison of mean cAKUSSI scores estimates at baseline and months 12, 24, 36 and 48 (*p<0·05) (Error bars represent ±95%CI).~~

~~Figure 3a – cAKUSSI scores over time (FAS)~~



~~Graph shows mean (95% CI) for baseline and adjusted mean (95% CI) for later time points.~~

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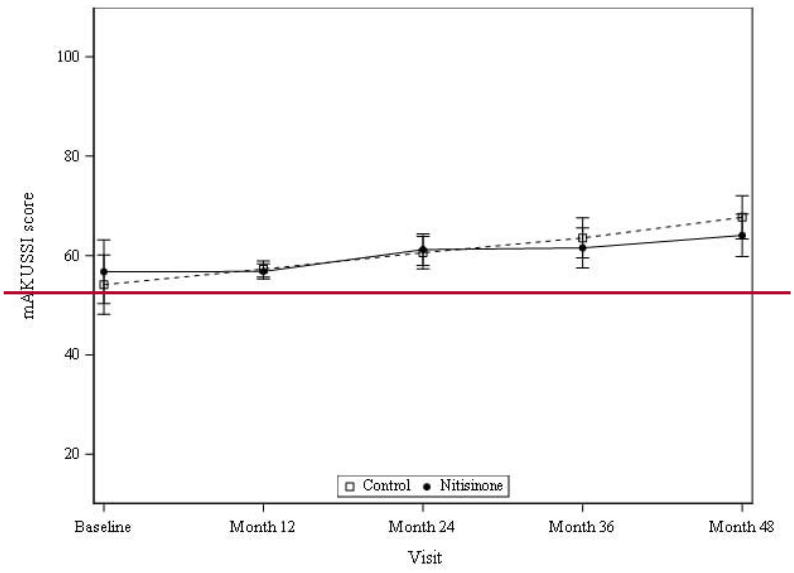
~~Figure 3b – mAKUSSI scores over time (FAS)~~



~~Graph shows mean (95% CI) for baseline and adjusted mean (95% CI) for later time points.~~

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Responses to Editor and Reviewers

We wish to thank the Editor and the reviewers for their helpful remarks. We have tried to address the issues raised in the sections below after careful and thoughtful consideration. In the interest of minimising increase in word count, added text is precise and relevant.

Page numbers are shown as MM followed by page number to indicate that this was in the main manuscript (**MM**). Similarly numbering for supplementary pages are shown as **SF** followed by page number. Files shown as **ADF** refer to additional data files.

CHECK ALL MANUSCRIPT & SUPPLEMENTARY PAGE NUMBERS.
SUPPLEMENTARY TABLES & FIGURES NUMBERS TO BE CHECKED.
RECHECK MANUSCRIPT PAGE NUMBERS AGAIN

Editors comments

Thank you for submitting your paper to The Lancet Diabetes & Endocrinology. Following a discussion with the editorial team, I am pleased to tell you that we have decided to invite you to submit a revised version of your manuscript that addresses the editors' and reviewers' comments below. We usually ask that authors of research papers return their revised manuscript within 2 weeks. If you cannot make this deadline, please let me know by email as soon as possible.
In your point-by-point responses to the reviewer and editorial comments, please state the page number and paragraph of the manuscript where changes have been made as a result. It can be helpful to tabulate your responses with columns labelled (left to right) as follows: Reviewer comments; author response and changes made; page number in revised paper where the change can be found. Please provide a 'clean' version of the manuscript, incorporating changes, and a 'tracked' changes version (highlighting additions and deletions - please use the 'Track changes' function in Word). IMPORTANT: where a reviewer has asked for clarification, it is usually necessary to amend the manuscript as well as answering the question directly in the point-by-point response document - where no changes have been made to the manuscript, please provide justification. I look forward to hearing from you.

Comments	Responses and changes	Page number where change found
1. When you upload your revision, please include a completed CONSORT checklist as necessary additional data.	Completed and submitted	
2. Please include the Abstract in the main article.	Completed	MM Page 3, 4
3. Please include the Research in context panel in the main article after the Abstract.	Completed	MM Page 5, 6
4. Please include a Contributions section at the end of the article detailing the contribution made by each author to the article.	Completed	MM Page 22
5. Please include a Declarations of interest section, after the Contributions section, listing the conflicts of interest of all authors. Note that any conflicts declared must match exactly those declared on the individual ICMJE forms.	Completed	MM Page 23
6. Please have each author complete an ICMJE form and upload these forms as Companion files when you submit your revision.	Completed and pdfs will be uploaded as additional data	Provided as ADF
7. Please have each author sign a completed Author signature form and upload as Companion files when you submit your revision.	Completed and pdfs will be uploaded as additional data	Provided as ADF
8. Please include an Acknowledgements section at the end of the article, if appropriate. Note that for any persons named in the Acknowledgements section, we need email confirmation from those individuals stating that they are happy to be acknowledged in the article. These email confirmations should be uploaded as Companion files.	Completed and pdfs will be uploaded as additional data	MM Page 24 Also Provided as ADF
9. Please include a Data sharing statement at the end of the article.	Accept	MM Page 25
10. Please provided editable figure files for each figure.	Done	UPLOADED
11. We usually have a limit of 6 display items for original research articles. You currently have 10: 3 Tables and 7 Figures. Please move Table 1 to the appendix. Please move Figure 1 to the appendix. Table 1 in the article should be baseline characteristics (currently Table 2). Figure 1 in the article should be the CONSORT flow chart (currently Figure 2). Please consider if any of the other figures can be moved to the appendix.	We have also moved Figures 1, 5, 6 and 7 to the supplementary (now Figures S2, 3, 4 and 5). Previous Table 2 is now Table 1 (Baseline characteristics). We have changed the design and title of previous Table 1, now numbered Table 2 in the main manuscript, as we feel that the results of the main primary and key secondary end-points should be presented in the main manuscript and not in the supplementary. We now have three tables and three figures in the main manuscript.	Figure 1 becomes New Figure S2 – SF Page 9 Figure 2 becomes New Figure 1 - MM Page 31 Figure 3 becomes New Figure 2 – MM Pages 32, 33

		Figure 4 becomes New Figure 3 – MM Pages 34, 35 Figures 5, 6 and 7 become New Figures S3, S4 and S5 S3 – SF Pages 10, 11 S4- SF Pages 12,13,14 S5 - SF Pages 15, 16
12. Please merge Table 3 with Supplementary Table S6 (as asked for by the statistical reviewer) to show all adverse event data - this should now be Table 2 in the article. All AE data discussed in the article should be included in this new table, including the ocular events. No AE data should be discussed in the article without it being included in the Table of AEs. Note that all AEs should be reported as total number, and broken down by degree of severity: mild, severe, and serious, and then broken down by class, then broken down by type.	These are two entirely different tables. While Table S6 gives an overview of number of AEs, SAEs, etc., Table 3 shows the most common SOCs for the AEs. We do not see the how merging the two can be easily done or improve the presentation. We suggest instead that we include Table S6 in the main paper to replace the old Table 3. We have included this now (Table S9). We have included this now.	NA Please see tables S9, S10, S11 and S12 Found in SF pages S9 – SF Page 29 S10 – SF Page 30 S11 – SF Page 41 S12 – SF Page 52 This is now Table S12 – SF Page 52
13. When you submit your revision, please include all files.	Accept	NA
Reviewer 1		
1. Can the fact there was an interim analysis please be stated in the paper	Sentence added under Study design. Please note that for the primary outcome, the 1-year analysis was not an interim one.	MM Page 9
1.a. In the protocol it says that this was for a regulatory filing and the trial would continue to approval of the treatment by the regulatory agency or four years. Can it please be stated of the treatment has been approved or not	An application for approval of the new indication was filed, based on the 4-year data, by Sobi in February 2020. The 1-year interim analysis of secondary outcomes did not support a submission at that time. A statement about this has now been included.	MM Page 14
1b. Can it be confirmed that the study would have continued regardless of the results as stated in the protocol	Yes, it would have continued, since only the EMA could judge if the results after 1 year could have been acceptable for an approval of the indication. We find it unnecessary to state that we follow the protocol. Furthermore, as stated above, the results after 1 year did not support a filing at that time.	Stated on MM Page 9
1 c. Can it please be stated who did this analysis and the submission. If it was by members of the study team can it please be clarified how this may have impacted on the results	The statistical analysis was performed by the trial statistician at the University of Liverpool. It is important to keep in mind that a lot of people, including patients, staff at the investigator sites etc. were aware of which treatment each patient was receiving. We therefore do not think that this had any further impact on the results of the final analysis after 4 years. However, the results of the interim analysis were not communicated to investigators or patients, only to the team responsible for regulatory authority interactions and decision to submit a file or not. (Patients and investigators were informed about the HGA and tyrosine results only)	NA
1 d. Was there an analysis plan	Yes, provided as additional data file	Provided as ADF
2. Can you please complete a CONSORT checklist.	CONSORT Checklist completed and provided as additional data	Provided as ADF
2 a. There needs to be more detail on the sample size section (please see below)	As below.	
2b. There needs to be more on possible biases	Randomisation bias excluded as far as possible by rigorous attention to masking and concealment as described in the statistical section of the manuscript. We could not blind patients as already explained and therefore there is the possibility of bias due to patient expectations especially in relation to pain scores, quality of life questionnaires and possible active range of motion.	Added to supplementary file item S10.0, SF page 6

	Drop-outs were not followed up and it is unknown if any bias was introduced. Intention-to-treat analysis was followed as described a priori in statistical analysis plan. All data collected in drop-outs were analysed. Despite involvement of industry partners, every care was taken to minimise the effect of competing interests. The role of industry partners has been further described under reviewer 4 responses (response 4). We have declared all data including positive and negative results to minimise publication bias.	
2c. There needs to be a statement that a limitation is that the primary outcome is a surrogate and why chosen	The primary outcome can be considered a surrogate. However, contrary to many other surrogate outcomes (e.g. cholesterol for cardiovascular disease), HGA is more than a risk factor. It is the actual cause of the clinical symptoms in patients with AKU. Thus, preventing the accumulation of HGA will inevitably prevent AKU-related symptoms from appearing, or slow down the worsening of ongoing symptoms. This was realized by the EMA, who therefore suggested that we use HGA as the primary outcome. We are therefore reluctant to call this a limitation. The text has been amended to better explain this (Background section).	MM Page 7
2d. Can the study title please give the study design	Manuscript tile has now been amended	MM Page 1
2e. When quoting results in text can you please always give the confidence intervals for the treatment difference	This has now been addressed.	MM Results section
2f. Can more detail please be provided on the randomisation procedure. Who did it? What package was used? How was it accessed and by whom?	A few words have been added under the "Randomisation and masking" section.	MM Pages 9, 10
3. Can the CONSORT checklist for abstracts please be completed	Has been completed and shared as additional data file	Provided as ADF
3a. The abstract at the moment has a lot of results. The focus should be on the primary outcome (with 95% CI).	Suggested to remove the individual AKUSSI items and only leave u-HGA, cAKUSSI and safety data. For uHGA the treatment difference estimate along with confidence intervals were added next to the p-value which was already included.	MM Pages 3, 4
3b. Key secondary can be given with 95% CI. Given the study was powered on the outcome the suggestion would be Akussi	Modified	MM Pages 28, 29 (Table 2)
3c. Can the incidence AEs please be given as well as eye related adverse events	Information added under the 'Safety section'.	MM Page 15
4. Can the CONSORT checklist for HARMS please be completed	Checklist completed and provided as additional data.	Provided as ADF
5. In the analysis of the adverse events (and Table 3) a. In this study (p19) in the protocol there was anticipated to be ocular adverse events. For individual AEs it is good practice to divide them into two i. Anticipated AEs ii. Study emergent AEs	Table 3 has been amended as suggested. This is now Table S11.	SF Page 41
5b. For the former I would report them even if there are zero events observed. I would also provide 95% for the difference between treatments (the summary data are in text but not in (Table 3). For the latter I would just provide summary statistics (as per Table 3)	We have reported unanticipated AEs even when zero events were observed as suggested and provided p-values for the anticipated AEs that compare the number of patients reporting those between the two arms. Please note that this is now Table S11.	SF Page 41
5c. Can Table 3 please be merged with Table S6. For the proportion rows in S6 I would have 95% CI for the difference between treartments	See comment on Page 1 No.12. We suggest instead that we include old Table S6 to replace the old Table 3 in the main manuscript.	MM Page 30
5d. There is a separate section in the paper on ocular adverse events and so as these were anticipated they should be tabulated in more detail	A new Table S9 is now inserted in supplementary file.	SF Page 29
5e. Any other anticipated adverse events should be given as rows in Table 3	All anticipated AEs are shown in Table S11.	SF Page 41
5f. There is no mention on whether tyrosine levels were associated with adverse events. The	For logistical reasons, the keratopathy cases did not have serum tyrosine measured at the time of keratopathy. Serum tyrosine was only checked	MM Page 18

protocol goes into quite a lot of detail on this. This can be complemented with supplemental material	during the baseline and annual study site visits. However, serum tyrosine was above 500 µmol/l in all treated patients, and levels were not higher in those who developed keratopathy than in those who did not. For each patient with keratopathy, the last tyrosine value measured while still on the 10 mg dose has been included in the keratopathy Table S9. A comment has been added in the text under safety section where this is discussed.	 SF Page 29 MM Page 18
6. Can you please complete the DELTA2 sample size checklist recently published and given in Table 2 in (https://doi.org/10.1136/bmj.k3750).	We have reviewed the BMJ guidance and we thank the reviewer for sharing this. There is only one previous randomised nitisinone trial, so data is limited. Further the previous RCT was inconclusive. The previous RCT used a different outcome, not applicable to SONIA 2. Therefore, the DELTA2 method was not used for SONIA 2 as it would not have been possible.	
6a. Can it please be explicitly stated here and throughout the paper that the primary outcome is a surrogate endpoint as stated in the protocol.	See our response to 2c above. We have now mentioned this once in the text but see no need to repeat this.	MM Page 7
6b. At the moment there is insufficient detail in the paper as to the justification for powered on the secondary endpoint (and indeed which AKUSSI endpoint cAKUSSI or mAKUSSI)	The text has been amended under the "Statistical analysis" section.	MM Page 11
6c. An effect size of 0.5 is quite large and effect to power on and this should be justified more	"While it is true that 70 subjects per arm would assume an effect size of 0.5, the least effect size that would result in statistical significance would be about the 0.3 which was deemed relevant. In addition, the use of MMRM rather than a plain student's t-test would allow even somewhat smaller observed effect sizes to be statistically significant." This figure was chosen given the profound decrease in HGA by nitisinone of greater than 99%. As a comparable example in statin trials where effect size of 0.3 is often used, the decrease of cholesterol is around 30 – 40%. This is clarification for the reviewer and text not added in manuscript	
6d. Of note of 107 publicly funded trials in the UK - https://doi.org/10.1186/s13063-018-2886-y - the average standardised difference planned for was 0.3 and very few had effects as large as planned for in this study. The trial was therefore aiming for a large effect and one that likely would have not been funded in the UK.	Very few medications have as profound an 'on-target effect' as nitisinone has in AKU, where a decrease of over 99% was observed for $uHGA_{24}$ and serum HGA. In comparison, in statin trials for cardiovascular disease prevention the decrease in cholesterol is typically around 30%. SONIA 2 is therefore unusual if not unique. The effect size was based on the assumption that increase in symptoms (AKUSSI score) could not be completely prevented with nitisinone, since all included patients were already in a phase of disease progression. But it was assumed that we could lower the progression to about one half of what has previously been seen in untreated patients. We had, however, no previous data, clinical or preclinical to guide us. This is clarification for the reviewer and text not added in manuscript	NA
6e. No comment is made that the sample size per arm is 69 per arm and not 70 (when talking about disposition of patients and Figure 2)	SONIA 2 was funded by the European Commission under their FP 7 programme, with a strict time limit for its performance. Therefore, we were forced to end recruitment after 138 included (139 screened) patients in order to fulfil our commitment. The fact that we managed recruit this many patients during a period of 9 months is still an enormous achievement in this very rare disease.	MM Page 13
7. For the statistical analysis 7a. There needs to be statement that there is no allowance for multiplicity	Sentence added under statistical analysis section.	MM Page 11
7b. For the statistical analysis a recent article in Pharmaceutical Statistics came out (https://doi.org/10.1002/pst.1964) with exemplar text for analysis such as in the paper. Can you please amend and apply as appropriate (there would not be too much changing) "Mean changes from baseline will be analysed using a restricted maximum likelihood (REML)- based repeated measures approach in	Thank you for recommending this interesting article. We have amended text under the 'Analysis of primary end-point' section. However, we have chosen not to include text on the alpha level and the statistical package used as it is already made clear under the 'Statistical analysis' section that a two-sided 5% level of significance is used throughout the analyses and all analyses were performed with the SAS system.	MM Page 11

combination with the Newton Raphson Algorithm. Analyses will include the fixed, categorical effects of treatment, investigative site, visit, and treatment- by- visit interaction, as well as the continuous, fixed covariates of baseline score and baseline score- by- visit interaction. A(n) common unstructured (co)variance structure will be used to model the within- patient errors. If this analysis fails to converge, the following structures will be tested in a subsequent order until model- convergence is achieved: (insert a list of structures appropriate for the specific application). (…) The Kenward- Roger approximation will be used to estimate denominator degrees of freedom. Significance tests will be based on least- squares means using a two- sided $\alpha = .05$ (two- sided 95% confidence intervals). Analyses will be implemented using (insert software package and analysis procedure). The primary treatment comparisons will be the contrast between treatments at the endpoint visit"		
7c. Can the statistical analysis plan please be provided	Accept and provided	Provided as ADF
7d. At the moment the analyses as presented do not represent the analyses as described (will go into more detail later)	See below	
7e. The protocol mentions using multiple imputation as a sensitivity analysis. Was this done? If so can it please be provided in supplemental	Tipping point analysis was performed primarily for regulatory purposes. As we do not feel that the reader of Lancet would easily comprehend this, we have decided to provide the sensitivity analysis results as data on file.	Provided as ADF
7f. Was any investigation made as to if the primary outcome was predicted of AKUSSI?	HGA is increased from birth and it is the cumulative effect of increased HGA that leads to the clinical picture that is measured in the AKUSSI, which increases with age. $uHGA_{24}$ is reflective of the increased HGA burden at any given moment. sHGA and $uHGA_{24}$ respond immediately to nitisinone treatment; both can also vary day to day with varying protein intake. Thus, these parameters cannot be correlated. No additional text added in manuscript – for reviewer's information only.	
7g. Can you please break the primary outcome down by the stratification factors in the randomisation (age and centre). This can be a Forest plot and can be supplemental	We have provided two new tables in the Supplementary material that address this. (Tables S6 and S7)	SF Pages 24-25 and 26-27
8. For the Tables 8a. For Table 2 i. This should be numbered Table 1 ii. It is redundant to have means and medians. Please use one only as appropriate iii. Do not need to give the sample size for all factors unless not as per the column heading iv. Do not need to give males and females (as one begats the other) v. Can the table have baseline outcome assessments (as in current Table 1) vi. Can the table please have centre	 Table number changed We have kept means as variables were normally distributed Removed Changed We do not think that this information should be given twice, both in what was Table 1 and in the demographics table (now Table 1) Added	 MM Page 27
8b. For Table 1 (as now) i. As stated above the baseline data should be in the demography table	 We do not agree. See above.	

ii. Can the title please state the analysis population	Yes, changed -Table 2. HGA and other continuous efficacy variables in AKUSSI	MM Pages 28, 29
iii. Personally I would keep this table just for 12 months and have the other time points in supplemental (there are still the figures to show effects over time in the main paper)	The strength of the current table is that it shows all metabolic and AKUSSI data unlike the figures and our strong preference would be to keep it. We have reduced the table, and kept the 48-month data as that was time for evaluation of the AKUSSI and no effect on clinical outcome was seen at Month 12 (kept baseline, M12 and M48).	
iv. The table must have an estimate of effect between treatments associated 95% CI	Addressed	Done
v. uHGA must be presented as analysed with Geometric Means (and CVs in brackets) with Geometric mean ratios	Addressed	Done
vi. I assumes Tyr and sHGA were also logged for analysis	Yes, s-TYR also now logged and text clarified and added under the "Analyses of other endpoints" section in main manuscript	MM Page 12 SF Page 19 (Figure S8)
vii. Mention is made of the results from SONIA 1?. Does the CI of the results from this study contain the effect observed in this study? It would infer the results are consistent and the original study was maybe too small (as stated in the introduction)	We think there has been a misunderstanding about the two studies, the SONIA 1 which was our own dose-response study, and the efficacy study by Introne et al. It is the latter that is considered too small to show an effect on a single clinical outcome (hip rotation). In SONIA 1, we found that the 8-mg dose reduced u-HGA24 to 326.7 µmol (CI -425.8 to 1079.3 µmol) after 4 weeks in 8 patients.	
viii. Can all outcomes as per p25 in the protocol please be in this table. At the moment there are no quality of life outcomes	We have done this as shown in Table 2. The updated table now provides the adjusted mean difference along with 95% confidence intervals and associated p-values that will make the interpretation much easier, rather than stating summary statistics. We hope that the results all clear now as suggested by the reviewer.	MM Pages 14, 15 Also Table 2 Pages 28, 29
ix. cAKUSSI is looking odd. At 48 months there is an effect of 1.9 with and SD circa 37 with a P=0.02. At 24 months there is an effect of 8.3 with a smaller SD but P=0.89. At month 12 the effect is smaller than month 24 but with a smaller P-value	The cAKUSSI emphasises the waxing and waning nature of AKU as in Figure 3A. The untreated group show a linear increase but the treatment effect shows intermittent worsening. We think this is because patients feel better on nitisinone especially their mobility and in so doing trigger complications such as joint, bone and ligaments failures. These seem to be an issue especially in the first 24 months before stabilising. The divergence of the control and treatment curves is clearly seen and is consistent with a beneficial effect of nitisinone.	
9. I personally think too much text is given to explaining the results from the AKUSSI components. I would personally move the results from the components to supplemental	This has been amended now (eye and ear pigmentation deleted).	MM Pages 14, 15
10. For Figures		
a. Figures 3a-3b should be on the log scale for the y-axis and there should be CI too for Nitisinone.	New figures are now provided showing the log scale for y-axis. Please note that those two figures are now Figures 2A and 2B.	MM Pages 32,33
b. These figures need to have CI for both arms. There might be for both but the scaling is not allowing it to be seen.	There were indeed CIs included in the figures but not able to distinguish the nitisinone ones due to scaling.	Done
c. A Figure for mAKUSSI should be provided	We have now provided this figure. Please note that this is now the new Figure 3B.	MM Page 35
11. Throughout the paper do not say "significant" or "significantly". Use "statistically significant" and "statistically significantly". In places do this but in others do not	Amended	
12. For the DMC		

a. Can members of the DMC please be given in the acknowledgments b. Can frequency of meetings please be given and any amendments made at the request of the DMC please be stated c. Can it please be stated if they assessed efficacy at all or just looked at safety (relevant as there was an interim analysis) d. Can the DMC charter please be provided: not this is not a request need to provided as not in CONSORT and so Lancet do not specify need to provide (it soon will be in CONSORT though)	Yes – already included DMC meetings were held once yearly. No DMC review resulted in a protocol amendment. Only safety – the intention was always to complete the 4-year duration if safety was acceptable regardless of efficacy data at 12 months. The DMC charter reflects this decision. Provided – as additional data file	MM Page 24 SF Page 5 DMC Charter provided as ADF
13. Can the data handling procedures please be described: how extracted, how coded, database used etc	The data for the SONIA 2 trial has been collected using an electronic Case Report Form (eCRF). The eCRF is developed in Viedoc 3, a 21 CFR Part 11 compliant web-based software, based on the study protocol. The set-up was approved after User Acceptance Testing by Sponsor and CRO. Data was entered by the Principal investigators or designees. The completed data was checked on site with the source for completeness and accuracy. Central assessors, experts on specific examinations, reviewed specific tests and provided the results. These results were also captured in the eCRF. Principal investigators confirmed all data to be complete and correct by signing off all completed data. Medical History terms and Adverse Events were reported in the eCRF and coded with the MSSO MedDRA dictionary version 21.1. Concomitant medication was coded with the ATC codes from the WHO drug dictionary ATC/DDD Index 2019. All coding was approved by a Medical Doctor and Pharmacist. Data exports were provided to the Trial Statistician from the University of Liverpool in SAS format. Once the data extract was received by the UoL trial statistician, then any data handling and analysis was performed in SAS and datasets were stored in a secure UoL server.	SF Page 5
14. Can the reference number for the ethics committees please be provided	EC Liverpool (NRES Committee North-West – Liverpool Central) Reference number: 13/NW/0567 EC Piestany (NURCH Ethica Committee, National Institute of Rheumatic Diseases, Ivana Krasku 4,92101 Piestany, Slovak Republic) Reference number: 04196/0029/001/001 EC Paris (EC Ile De France II, hospital Necker 149 Rue de Sevres 75 743 Paris Cedex 15, Porte N2, 1er etage, France) Reference number: 2013-08-08	SF Page 5
15. Version 4 of the protocol has been provided. Can details please be provided on the amendments and any major amendments commented on in the main paper	The amendments mostly contained corrections and clarifications. There were no major changes that affected the performance of the study in any important way. Amendment 1 added results from SONIA 1 in the background and defined the dose. This information was missing when Version 1 was written. Amendment 2 only contained corrections and clarifications. In amendment 3, MRI was reclassified from a secondary to an exploratory assessment and changes to the study staff were presented, along with further clarifications.	SF Page 5
Edits 16. Please for all web references give the date last accessed	Checked and not found now and deleted. References renumbered.	MM Pages 20, 21
17. Can references 13 and 23 have more detail	Checked and amended. Further references added and references renumbered.	MM Pages 20, 21
18. Can you please be consistent with you spelling as to English (Randomised, Summarised) or American (Randomized, Summarized)	Amended as suggested.	
Reviewer 2		

The manuscript by Dr Ranganath and colleagues summarizes the results of an unfortunately rare event of excellently planned and performed clinical study in rare diseases. The specific disease investigated is alkaptonuria, in 1902 the first ever reported monogenic disease in humans. The objectives, design and concept of the multinational study are well described as are results, limitations and discussion points. Most importantly for the patients the results show a positive clinical response of this chronic and in the long-term debilitating disease.		
The only point I am missing is the discussion of the in my view possible additional therapeutic concept in individuals who experience significant side effects because of the raised Levels of tyrosine. We know from the treatment of tyrosinaemia type I with nitisone that the consequently highly elevated levels of tyrosine should and can be lowered by a specific diet in analogy to many other genetic defects of mino acid metabolism. Such a diet is very cumbersome for adults to be introduced to and to adhere to long term. I can therefore see the value of trying the treatment without the diet and to carefully monitor side effects. However, once side effects occur and even lead to this continuation of the principally working therapy in some individuals, even if the dose of the medication is lowered, these individuals should in my opinion be actively approached to stay on therapy and reduce the elevated levels of tyrosine by the by now more than 50 years old method of lowering accumulating metabolites by a specific diet.	A diet sheet describing the lower protein diet was provided at baseline and this was reinforced to all participants at ach study visit. More active dietary intervention was made impossible due to having recruited patients from all around Europe and Jordan, often lacking contact with local dietetic healthcare. This was not without precedent as the previous RCT carried out in the NIH was also in free-living patients with no active dietary intervention. Further, serum tyrosine measurement and management would have become extremely complex. The diet issue is not discussed in the SONIA 2 manuscript due to word count issues. However, the dietary management of tyrosine is managed actively in clinical practice in the National Alkaptonuria Centre in Liverpool and a recent publication on the experience of using nitisinone contains such advice as being employed in the centre and is referenced in this manuscript (Reference 14 in main manuscript; Judd et al. Nutritional status of people with alkaptonuria: an exploratory analysis suggests a protein/energy dilemma. JIMD reports.2019:1-16; DOI: 10.1002/jmd2.12084). The following text has been included in the manuscript under discussion of keratopathy. "In patients who develop keratopathies, plasma tyrosine levels should be monitored. A diet restricted in tyrosine and phenylalanine should be implemented to keep the plasma tyrosine level below 500 µmol/L. In addition, nitisinone should be temporarily discontinued and may be reintroduced when the symptoms have been resolved".	MM Page 18, 19
Reviewer 3		
The authors report a randomized trial of nitisinone for alkaptonuria patients. The study participants were not blinded due to effects of the drug that are visible. Where possible, observers were blinded. The study provides follow up for 4 years. The primary outcome was biochemical response. Secondary outcomes were clinical responses. This study follows a significant number of patients over 48 months which is a strength. Unfortunately, due to higher than expected number of drop outs, the study remains underpowered for some key clinical events. Biochemical changes are striking as for previous patients and there is a reduction in ochronosis. However, although there is a great deal of data presented on the clinical outcomes, the vast majority of these are not significant. There is a suggestion that there may be a reduction in spinal pain and tendon ruptures but further analysis needs to be done to be confident of this. Also, with the expected (keratitis) and unexpected (infections) increased risk of adverse events in the nitisinone group, an overall analysis of harm versus benefit as the evidence of clinical benefits are relatively modest and there is both expected and unexpected toxicity. It would be very helpful for clinicians who care for these patients to actually have these data analysed in such a way as to see if there is a net benefit or net harm. Either conclusion would be very helpful (and publishable). If there is a net harm, then we need to move away from this drug and look for other strategies to treat these patients. If there is a net benefit, then we need to get wider access to the drug for these patients. Unfortunately, the manuscript in its current form does not allow for such conclusions to be made. Hopefully, further analysis of the data from this relatively large study could actually be conducted to answer the question of nitisinone therapy in alkaptonuria one way or anther. **This is an important point raised and highlighted in green. We will plan a publication along the lines suggested to explore the balance between benefit and harm. The profound decrease in HGA, and the beneficial effect on clinical parameters outlined and discussed, makes us think that this outweighs the mild reversible keratopathy observed despite not engaging in dietary protein restriction, and the minor unexpected increase in infection post-nitisinone. The key undesirable result of nitisinone is tyrosinaemia and should be overcome in clinical practice with real-time effective dietetic monitoring. The only alternative to inhibition of hydroxyphenylpyruvate dioxygenase to prevent formation of HGA (as exemplified by nitisinone) is homogentisate 1,2 dioxygenase replacement; it is the view of the authors that it seems highly unlikely such replacement therapies could be as effective in decreasing HGA, although tyrosinaemia is likely to be less of an issue. It is also not known if HGD-replacement therapy would also lead to infections in AKU. If HGA control is less effective, morbidity outcomes may not be as positive as with nitisinone.**		
Major 1. The authors need to provide more information for the readers as to why they choice a different dose than that was studied in the previous randomized trial given that the biochemical response is also good for the lower dose and readers may not be familiar with the dose ranging studies	The dose used in the NIH trial was 2mg daily. Because the NIH was inconclusive, the issue of optimum dose was revisited in SONIA 1. The 8 mg dose in SONIA 1, rather than 1, 2 or 4 mg decreased to 'near-normal' in all subjects in the group. The EMA had indicated that 'normalisation' of $uHGA_{24}$ should be used as the primary outcome. Hence the 10 mg dose, closest to the 8mg, was chosen for SONIA 2, to maximise the chances of a successful study. Text in manuscript has been edited.	MM Page 8
Major 2. Unfortunately, because of a higher than expected drop-out rate, the study remains underpowered. The drop outs were mostly for A/E in the treatment group which is not unexpected. However, due to the higher drop-	We do not entirely agree that the study was underpowered. Given the use of MMRM for the longitudinal analysis all patients with at least one post-baseline assessment were always included in all the analyses. However, the accuracy for MMRM can of course be impacted by the missing data occurring from patients dropping out.	MM Page 11

out rate, it would be helpful to know if there are quality of life data available as the benefits of the therapy were largely nonsignificant (see below) and the predictable toxicity was limiting in a significant number of patients. This combination of effects then combine to limit effect on QoL.	We did see positive trends for SF36 especially up to month 36, and information about this has now been added to the text.	MM Page 15
Major 3. The authors report a statistically significant change from baseline in the cAKUSSI score at month 48 in table 1. This difference seems to be largely driven by the difference in baseline scores (higher in the nitisinone group) and, when looking at the figure where these data are graphed, these curves are largely overlapping albeit with the suggestion that they may be diverging later in the study. The graph though makes the data seem very unconvincing. If the change in score is graphed rather than the absolute score, this be more appropriate. Major 3a. It also would be helpful to know what this numerically small difference translates in terms of clinical relevance	Despite the slightly older group with more severe disease at baseline, the expectation is that the rate of change would be similar in the control and nitisinone groups. It is for this reason that we compared various time points between the control and nitisinone groups. The difference in cAKUSSI includes changes in all items included and is expected to include significant changes relevant to patients.	
Major 4. The authors report a statistically significant change from baseline in T score at 48 months between the 2 groups; however, the T score curves which are presented in the figures essentially are overlapping with no evidence that the curves are diverging and it seems unlikely that this would be a clinically relevant endpoint and the text should indicate this	BMD is an important predictor of fractures. In Bisphosphonate fracture prevention studies the BMD improvements reported are around 5% usually over 4 years and considered to be relevant to fracture prevention. In SONIA 2, the change in T-scores was -11.9% for the control group at 48 months compared to baseline and +6.1% at 48 months compared to baseline in the nitisinone group. A further reference has been added explaining this (Ref 23). It is emphasised that in SONIA 2 patients ranged in age from age 26 years to 70 years; it is likely that BMD varied significantly as a result.	No additional text added due to word count issues
Major 5. The fracture curves are difficult to interpret as the total number of fractures is presented rather than the rate of fractures per patient year. For example, in looking at the curves months 12-36, they are parallel and it appears that there is one more fracture (23) in the control group rather than 22 in the nitisinone group. At month 48, there is a spike in the control group. Given the drop out rates, we cannot infer that the number of patients at risk of fractures is uniform at the given time points and the analysis should be redone to look at the rate of fractures as a function of person years at risk rather than the cumulative number of fractures. Also, in the discussion, the authors state that there was a trend towards fewer fractures in the nitisinone group than in the control, in keeping with the statistically significant difference in change from baseline, in BMD, between the treatment groups. The absolute difference in BMD T-scores in the control group was 0.15 and the T scores remained above the osteoporotic range. It is unlikely that this small difference would result in any detectable fracture difference in a small study (although, in a large population based study with thousands of people, you may be able to see such a difference) and, in fact, many studies have shown that the change in T score over time does not predict fracture risk any better than the baseline (for example, see Leslie et al J Clin End Metab 2012 and others).	Please see response above. We believe that despite the small T-score absolute values, as the reviewer has suggested, in a larger number of patients with a homogeneous osteoporotic population, the fracture data would likely have been more obviously statistically significantly different.	
Major 5a. This same suggestion could be made for the tendon data - although this does	Please see the above response which is also relevant to the data on tendon ruptures, as this cohorts were not enriched in tendinopathies.	

look a bit more convincing graphically than the fracture data (in that the nitisinone group does appear to be flat), we need to have this expressed as a function of person years at risk		
Major 6. The joint pain analysis is also difficult to interpret. Although the authors have clearly stated that there is not a significant difference between the 2 groups (and that is also shown in the figure), assessing the total number of joints with pain may not reflect what is actually happening. What does the analysis shown when it looks at joint pain as change from baseline rather than total number of joints with each patient as his own control rather than aggregate?	We decided to compare control and nitisinone groups at various time points against each other rather than comparing change scores from baseline for each separately. The reason was that despite the slightly older group with more severe disease at baseline, the expectation is that the rate of change would be similar in the control and nitisinone groups. It is for this reason that we compared various time points between the control and nitisinone groups.	
Major 6a. For both the analysis of joint pain and spine pain, some form of discussion as to what is a clinically relevant change is needed (for example, what change correlates with a reduction in pain interference scores or reduced need for analgesic etc)	The difference in pain between the control and treatment groups could explain the beneficial difference in SF36 and range of motion between the two groups. This comment has been added to the discussion.	MM Page 18
Major 7. There was a higher incidence of infections in the nitisinone group - why is this? This is a significant finding when considering the ….	This was unexpected and the reason unknown. This has not been reported in HT-1 nitisinone usage of over 29 years – therefore probably not a direct nitisinone drug effect.	MM Page 18 – already discussed
Minor points Minor 1. Demographics and baseline characteristics (table 2) should go before the outcomes table (table 1)	This has now been addressed.	MM Page 27
Minor 2. Table 1 is confusing to read with the p values below each set of numbers and could be reformatted	We have now amended this table to make it clearer. The letter 'E' has been clarified.	MM Pages 28, 29
Minor 3. P values should be provided for Table 3 to easily distinguish which of these were more common in treated group rather than requiring people to refer to the text for that information; also the use of the term "E" for number of events is a bit confusing so it would be helpful if this variable is fully spelled out in the top of the table rather than just in the key to the table	The letter 'E' has been clarified. We decided to provide p-values as we thought these will be easier for the Lancet reader to understand rather than providing 95% CIs, for the anticipated AEs as per the reviewer's 1 request 5b.	MM Pages 28, 29
Reviewer 4		
Ranganath and colleagues report s study that is long waited for in the metabolic community. The paper is very well written and the message is clear and convincing, despite the strong involvement from industry. Accordingly, I have only few minor points and would like the authors to consider whether some minor revision would improve the manuscript further:		
1. The dose of nitisinone used (10 mg daily) is much higher than in previous Trials (2 mg); I did not find an exact justification for chosing this dose. Please add.	We have discussed this in some detail in responding to reviewer 3 under Major 1.	
2. The dietary aspects are unfortunately only very superficially considered in this study. For instance, while tyrosine levels were monitored, no complete amino acid profiles were done. However, this would have been informative especially for the nitisinone group in which some patients may have performed a self-selected low-protein diet. Now that the study is done, it may be too late to measure also other amino acids, especially the essential amino acids, in order to know better about the extent of the low-protein diet. Given the inevitable tyrosinemia that patients will experience when treated with nitisinone, this would have been a valuable additional information and useful for future treatment. Clearly for future studies with	Reviewer 2 also raised this issue and this aspect was discussed in some detail.	

nitisinone, a detailed dietary protocol should be considered.		
3. The power analysis for this study resulted in 140 patients minus an expected 10% drop-out. Now, only 108 patients completed the study. Authors should comment on this and whether results are still relevant.	Reviewer 3 also raised this issue and this was extensively discussed for Major 2.	
4. Can authors specify the contribution from authors from Swedish Orphan Biovitrum AB?	DevelopAKUre was a research consortium including both academic, industry and patient organisation partners. The contributions of each co-author are defined in the relevant section of the manuscript, and co-authors were only invited based on fulfilling ICMJE authorship criteria. Although Sobi is an industry partner in the consortium, the members were only within scientific functions, and not commercial, bringing vast knowledge and experience from global clinical development programs, design, analysis and research meeting regulatory requirements as well as scientific knowledge on nitisinone. The scientific integrity of the work was never compromised.	
Additional changes made to improve or correct errors		
Study limitations	This has been updated and amended. Extra references have been added (Refs 23, 27)	MM Page 19 MM Page 21

Tables

Table 1. Demographic data and baseline characteristics (FAS)				
Variable	**Statistic**	**Control (n=69)**	**Nitisinone (n=69)**	**Total (n=138)**
Age (years)	Mean (SD)	47·6 (10·1)	49·0 (11·3)	48·3 (10·7)
Body weight (kg)	Mean (SD)	74·1 (15·6)	74·8 (14·8)	74·4 (15·1)
Height (cm)	Mean (SD)	167 (9·5)	166 (9·2)	167 (9·4)
Sex n (%)	Male	40 (58·0)	45 (65·2)	85 (61·6)
Race n (%)	White	67 (97·1)	67 (97·1)	134 (97·1)
	Black	0 (0·0)	1 (1·4)	1 (0·7)
	Asian	2 (2·9)	1 (1·4)	3 (2·2)
Centre n (%)	Liverpool	21 (30·4)	20 (29·0)	41 (29·7)
	Piešt'any	32 (42·6)	33 (47·8)	65 (47·1)
	Paris	16 (23·2)	16 (23·2)	32 (23·2)

Table 2. HGA and other continuous efficacy variables in AKUSSI (FAS)

		Baseline		Month 12		Month 48	
HGA							
HGA		Control	Nitisinone	Control	Nitisinone	Control	Nitisinone
u-HGA24 µmol	Mean (SD)	35394 (13869)	35019 (13124)	26444 (10397)	179 (398)	33207 (10160)	1569 (6220)
	Adjusted geometric mean (quotient nitisinone/control) with 95% CI	NA		0.003 (0·003 - 0·004)		0.005 (0·003 - 0·008)	
s-HGA mmol/L	Mean (SD)	28·26 (8·66)	30·35 (10·98)	28·93 (13·04)	0·71 (1·63)	37·08 (21·03)	2·80 (7·33)
	Adjusted geometric mean (quotient nitisinone/control) with 95% CI	NA		0.01 (0·01 - 0·02)		0.02 (0·02 - 0·03)	
AKUSSI							
cAKUSSI (points)	Mean (SD)	80·5 (33·4)	87·0 (34·2)	80·1 (34·7)	84·5 (33·7)	95·6 (36·0)	93·7 (37·8)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-2·5 (-5·7; 0·7)		-8·6 (-16·0; -1·2)	
mAKUSSI (points)	Mean (SD)	54·1 (24·9)	56·7 (26·7)	54·8 (25·7)	57·5 (26·8)	66·7 (29·7)	66·1 (31·1)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·5 (-2·5; 1·6)		-3·6 (-9·6; 2·4)	
Individual AKUSS items							
Eye ochronosis	Mean (SD)	14·1 (9·6)	17·3 (9·2)	14·7 (9·0)	16·8 (9·5)	16·4 (9·5)	16·5 (9·3)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·8 (-1·9; 0·3)		-2·5 (-3·9; -1·0)	
Ear ochronosis	Mean (SD)	3·9 (2·9)	4·1 (2·9)	4·0 (2·8)	4·1 (2·9)	4·0 (2·8)	4·0 (2·9)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·2 (-0·4; 0·0)		-0·5 (-0·9; -0·1)	
BMD (T-score)	Mean (SD)	-1.26 (0·98)	-1·3 (1·2)	-1·28 (0·98)	-1·39 (1·14)	-1·41 (0·81)	-1·22 (1·17)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·09 (-0·18; -0·01)		0·14 (0·00; 0·28)	
Aortic velocity (m/s)	Mean (SD)	1·6 (0·6)	1·8 (0·8)	1·6 (0·6)	1·8 (0·8)	1·7 (0·6)	1·8 (0·8)

	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·009 (-0·092; 0·075)		-0·030 (-0·149; 0·089)	
Joint pain	Mean (SD)	4·6 (3·3)	4·8 (3·0)	4·0 (3·1)	3·5 (2·7)	4·2 (3·3)	3·8 (2·7)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·9 (-1·6·; -0·1)		-0·7 (-1·6; 0·1)	
Number of joints with osteoarticular disease	Mean (SD)	6·7 (3·2)	6·1 (3·1)	6·7 (3·2)	6·4 (3·2)	9·1 (3·3)	8·5 (3·6)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		0·0 (-0·1·; 0·2)		-0·1 (-1·3; 1·1)	
Spinal pain	Mean (SD)	2·3 (1·2)	2·3 (1·3)	2·0 (1·2)	1·9 (1·3)	2·2 (1·4)	1·7 (1·3)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		-0·2 (-0·5·; 0·2)		-0·5 (-0·9; 0·0)	
Number of spinal regions with osteoarticular disease	Mean (SD)	3·0 (2·1)	3·4 (2·1)	3·0 (2·1)	3·4 (2·2)	3·5 (2·1)	3·7 (2·0)
		NA		0·0 (-0·3·; 0·4)		-0·1 (-0·4; 0·3)	
Kyphosis (Cobb angles)	Mean (SD)	35·2 (10·2)	36·4 (10·6)	35·2 (9·2)	37·1 (10·7)	37·2 (7·8)	39·5 (9·7)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		0·9 (-0·3·; 2·1)		1·0 (-0·8; 2·7)	
Scoliosis (Cobb angles)	Mean (SD)	10·5 (5·4)	10·8 (5·2)	10·5 (4·9)	10·7 (4·5)	11·8 (6·6)	12·1 (5·2)
	Adjusted mean (difference nitisinone-control) with 95% CI	NA		0·0 (-0·6·; 0·6)		-0·1 (-1·6; 1·4)	

NA: Not applicable

Table 3. Overall summary of adverse events (Safety analysis set)

	Control (n=69)		**Nitisinone (n=69)**	
	n (%)	**Incidence rate per patient years**	**n (%)**	**Incidence rate per patient years**
No. of patients with at least one AE	57 (82·6)	2·1	59 (85·5)	2·3
No. of AEs	284		400	
No. of patients with at least one SAE	26 (37·7)	1·0	27 (39·1)	1·0
No. of SAEs	52		57	
No. of patients with at least one related AE [a]	NA	NA	18 (26·1)	0·7
No. of related AEs	NA		48	
No. of patients who died	0 (0·0)	0·0	2 (2·9)	0·1
No. of patients with AEs leading to study discontinuation	1 (1·4)	0·0	9 (13·0)	0·3
No. of patients with AEs leading to dose reduction	NA	NA	8 (11·6)	0·3

[a] Related to study drug, as judged by the investigator.
AE: Adverse Event n: Number of patients observed NA: Not applicable SAE: Serious Adverse Event
Percentage calculated on n (patients in treatment groups)

Figure 1. SONIA 2 (CONSORT) Flow Diagram

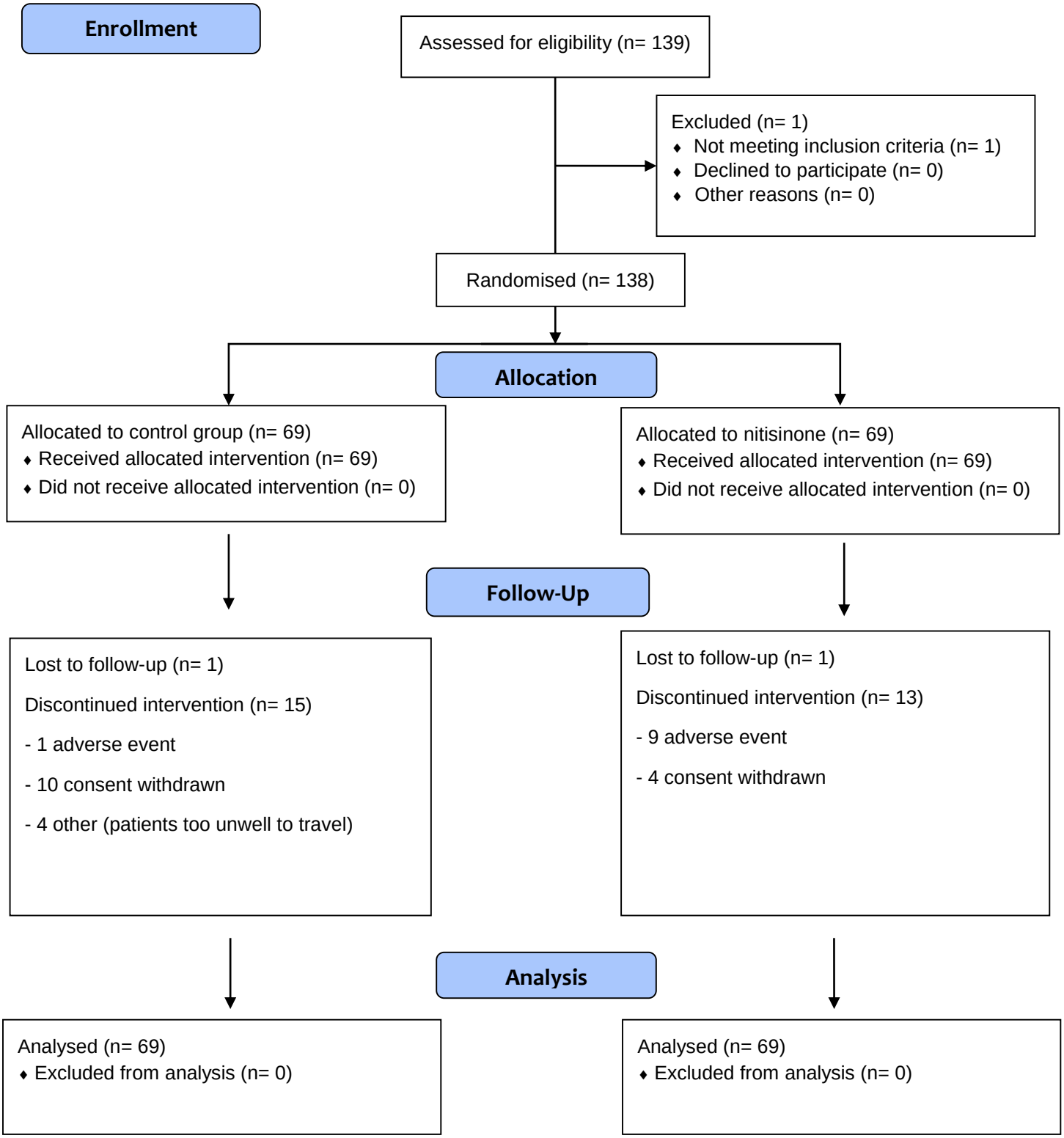
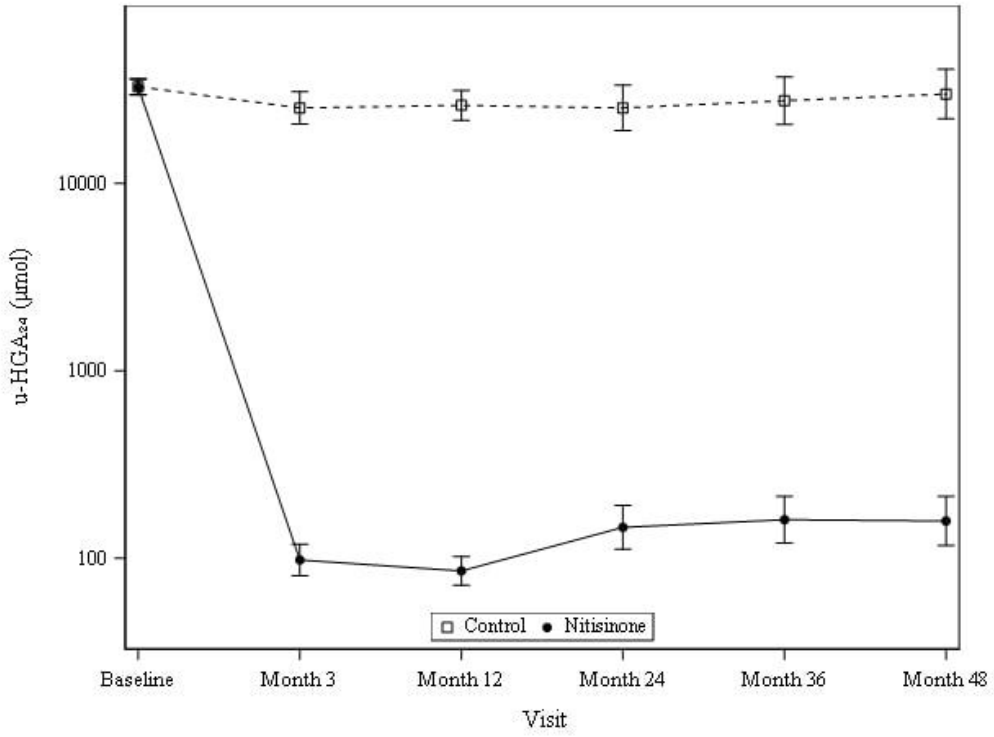
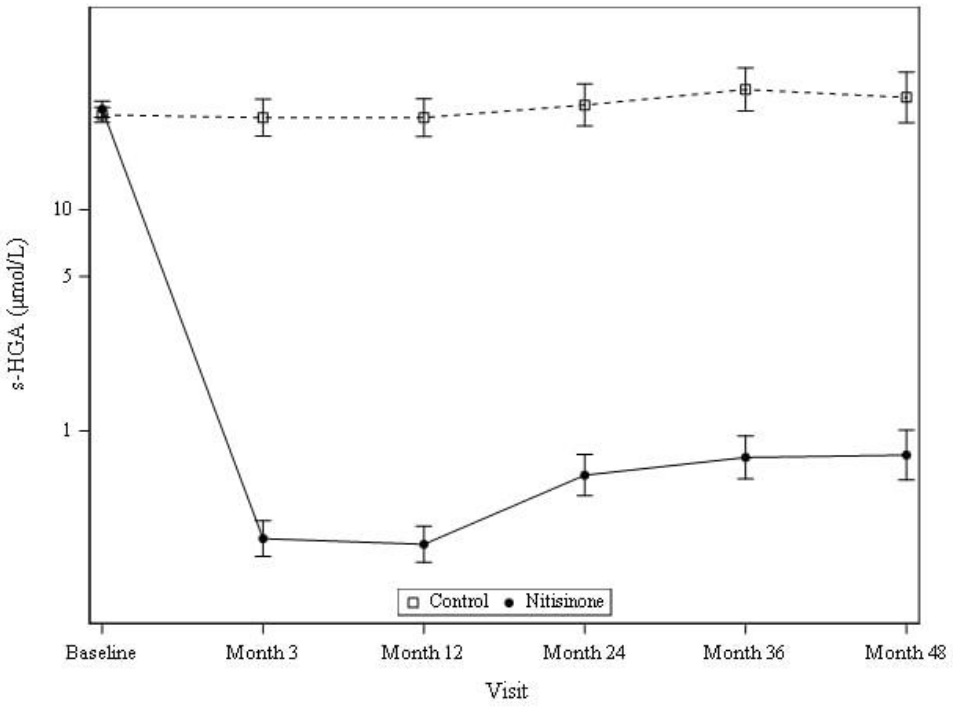


Figure 2A. u-HGA 24 (µmol) over time (FAS)



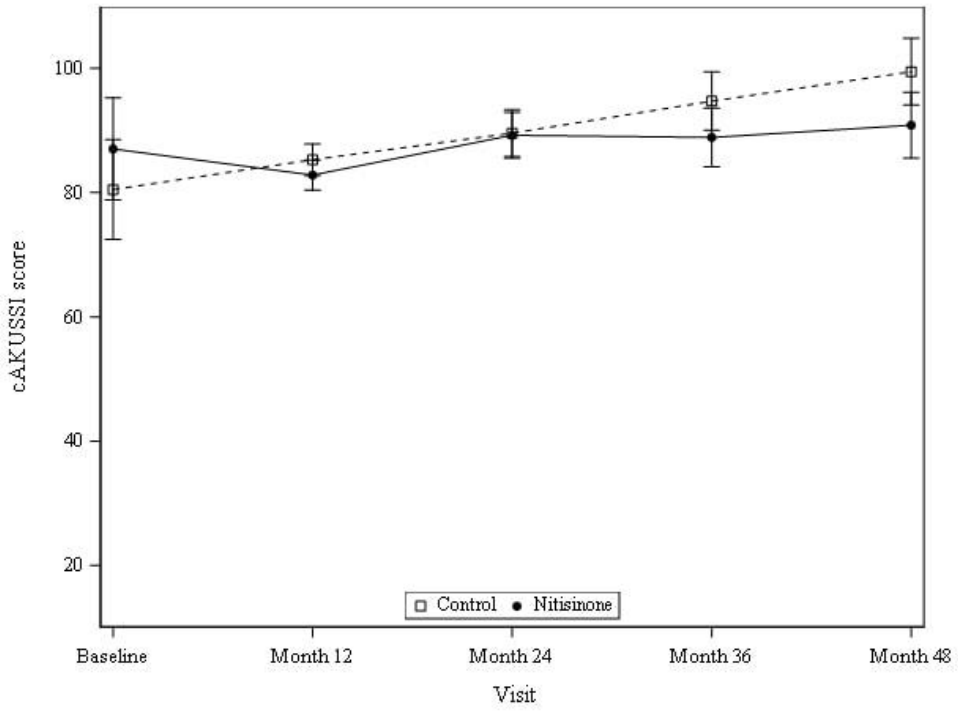
Graph shows geometric mean (95% CI) for baseline and adjusted geometric mean (95% CI) for later time points;
Y-axis on log scale.

Figure 2B. s-HGA (µmol/L) over time (FAS)



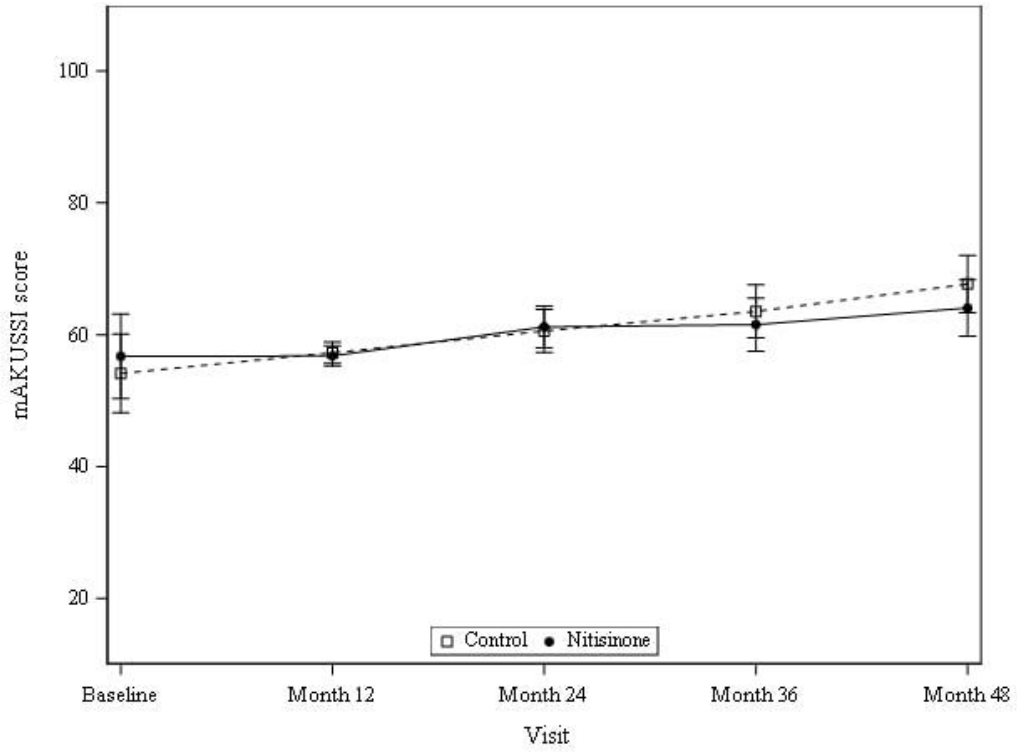
Graph shows geometric mean (95% CI) for baseline and adjusted geometric mean (95% CI) for later time points;
Y-axis on log scale.

Figure 3A. cAKUSSI scores over time (FAS)



Graph shows mean (95% CI) for baseline and adjusted mean (95% CI) for later time points.

Figure 3B. mAKUSSI scores over time (FAS)



Graph shows mean (95% CI) for baseline and adjusted mean (95% CI) for later time points.

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Sensitivity/tipping point analysis

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Necessary Additional Data

Figure 10.5 - 4.rtf

Sensitivity/tipping point analysis

Sensitivity/tipping point analysis

Sensitivity/tipping point analysis

Sensitivity/tipping point analysis

Sensitivity/tipping point analysis

Sensitivity/tipping point analysis

Sensitivity/tipping point analysis

An international, multicenter, randomized, evaluator-blinded, no-treatment controlled, parallel-group study to assess the efficacy and safety of once daily nitisinone in patients with alkaptonuria after 12 months of treatment, followed by an additional 36-month treatment period.

Final Protocol of the Study
SONIA 2

EudraCT no. 2013-001633-41

Type of Study: **Therapeutic confirmatory**

Co-ordinating Investigator

Professor Lakshminarayan Ranganath
Consultant in Clinical Biochemistry and Metabolic Medicine
Royal Liverpool University Hospital
lrang@liverpool.ac.uk

Signature Date

Medical Monitor

Dr. Helena Glasová, MD, PhD
Institute of Pharmacology, Slovak Medical University, Bratislava
helena.glasova@szu.sk

Signature Date

Statistician

Dr Trevor Cox
Senior Lecturer in Statistics
University of Liverpool
coxt@liverpool.ac.uk

Signature Date

Investigator statement

I have read the protocol entitled "An international, multicenter, randomized, evaluator-blinded, no-treatment controlled, parallel-group study to assess the efficacy and safety of once daily nitisinone in patients with alkaptonuria after 12 months of treatment, followed by an additional 36-month treatment period" and the accompanying Investigator's Brochure. I agree to conduct the clinical investigation in compliance with the Final Protocol, Version 4.0, dated July 22, 2016, the International Conference on Harmonisation (ICH) harmonised tripartite guideline E6(R1): Guideline for Good Clinical Practice, applicable regulatory/government regulations, and in accordance with the latest revision of the Ethical Principles for Medical Research Involving Human Subjects (the Declaration of Helsinki). I will not implement any changes to study procedures or conduct without prior approval from the DevelopAKUre consortium and, when applicable, the Independent Ethics Committee and Regulatory Authority.

I agree to maintain the confidentiality of this study protocol, as described on the title page. Further, I will not publish results of the study without authorization from the DevelopAKUre Consortium.

Signature of Principal Investigator	Date

Printed Name of Principal Investigator

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1 Synopsis

STUDY IDENTIFIERS

Title of study:	An international, multicenter, randomized, evaluator-blinded, no-treatment controlled, parallel-group study to assess the efficacy and safety of once daily nitisinone in patients with alkaptonuria after 12 months of treatment, followed by an additional 36-month treatment period.
Clinical acronym:	SONIA 2 (Suitability Of Nitisinone In Alkaptonuria 2)
Investigators:	Professor Lakshminarayan Ranganath Professor Jozef Rovenský Dr Jean-Baptiste Arnoux
Study centers:	Royal Liverpool University Hospital Trust (RLUH), Liverpool, UK National Institute of Rheumatic Diseases, Piešťany, Slovakia Hôpital Necker-Enfants Malades, Paris, France.
Type of study:	Therapeutic confirmatory.

STUDY OBJECTIVES

Primary objective: To demonstrate that nitisinone is superior compared to no treatment in reducing 24-hour urinary homogentisic acid (HGA) excretion in patients with alkaptonuria (AKU) after 12 months.

Secondary objectives:

- To demonstrate the effect of nitisinone on u-HGA_{24} after 3, 24, 36 and 48 months.
- To demonstrate the effect of nitisinone on control of serum HGA concentration (s-HGA) in patients with alkaptonuria after 3, 12, 24, 36 and 48 months of treatment.
- To demonstrate the effect of nitisinone on pre-defined clinical parameters.
- To assess the association between u-HGA_{24} and change in clinical parameters.
- To assess predose serum concentrations of nitisinone after 3, 12, 24, 36 and 48 months of treatment.
- To assess the association between predose serum concentrations of nitisinone and u-HGA_{24}.
- To assess the effect of nitisinone on pre-defined measures of health and functional status, as assessed by SF-36, HAQ and KOOS.
- To assess the safety of long-term treatment with nitisinone in patients with AKU.

Exploratory objectives:

- To explore the effect of nitisinone on inflammatory biomarkers, and biomarkers of bone, cartilage and cardiovascular damage.
- To explore the effect of nitisinone on metabolites of tyrosine (other than HGA).
- To explore metabolic pathways in AKU patients (metabolomics).
- To explore the effect of nitisinone on spine and joint disease as assessed by magnetic resonance imaging and knee radiographs.
- To explore the association between AKU genotype and phenotype.
- To explore the use of digital image analysis of photographs, X-rays and scans as a measure of disease progression of AKU.

STUDY ENDPOINTS

Primary endpoint: u-HGA_{24} after 12 months.

Secondary endpoints:

- u-HGA_{24} after 3, 24, 36 and 48 months.
- Occurrence of achieved target level (< 300 µmol) of u-HGA_{24} at 3, 12, 24, 36 and 48 months.
- Predose s-HGA at 3, 12, 24, 36 and 48 months.
- Change from Baseline in Clinical AKUSSI scores at 12, 24, 36 and 48 months.
- Change from Baseline in Modified AKUSSI scores at 12, 24, 36 and 48 months.
- Change from Baseline in individual cAKUSSI items at 12, 24, 36 and 48 months.
- Change from Baseline in ear cartilage pigmentation at 48 months.
- Change from Baseline in range of joint and spine motion at 12, 24, 36 and 48 months.
- Change from Baseline in other pre-defined rheumatology assessments at 12, 24, 36 and 48 months.
- Change from Baseline in quality of life (QoL) measured by SF-36 at 12, 24, 36 and 48 months.
- Change from Baseline in health assessment measured (including pain scores) by HAQ at 12, 24, 36 and 48 months.
- Change from Baseline in physical function as measured by KOOS at 12, 24, 36 and 48 months.
- Pre-dose serum nitisinone at 3, 12, 24, 36 and 48 months.
- Adverse events, s-Tyr, clinical chemistry and hematology, vital signs, ECG and corneal eye assessments.

Exploratory endpoints:

- Biomarkers of inflammation at Baseline.
- Biomarkers of bone, cartilage and cardiovascular damage at Baseline.
- Biomarkers of inflammation and of bone, cartilage or cardiovascular damage which were elevated at Baseline, at 3, 12, 24, 36 and 48 months.
- Tyrosine metabolites other than HGA.

- Metabolomics.
- Measures of spine and joint disease.
- AKU genotype.
- Digital image analyses of photographs, X-rays and scans.

STUDY DESIGN AND METHODS

Study design:	A randomized, open-label, evaluator-blinded, parallel-group study with a no-treatment control group.
Number of patients:	140 (70 per group).
Diagnosis and main criteria for inclusion:	Any clinical manifestations of AKU, such as clinical ochronosis or chronic back / joint pain and at least 25 years of age.
Assessments:	• 24-hour urinary HGA excretion, serum HGA, tyrosine and nitisinone. • AKU Severity Score Index. • Ear cartilage biopsy. • Range of motion of spine and joints. • Functional health and QoL surveys. • Safety assessments. • Urine and serum biomarkers for inflammation and bone, cartilage and cardiovascular remodeling. • Metabolomics. • Magnetic resonance imaging of spine and target joints. • Knee radiographs. • AKU genotype.
Test product; dose and mode of administration:	Orfadin capsules containing 10 mg nitisinone, administered orally once daily.
Reference product; dose and mode of administration:	None.
Duration of treatment:	48 months.
Statistical methods:	The primary analysis will be a mixed model repeated measurement (MMRM) as follows: log(u-HGA_{24}) = treatment, site, age category, visit, treatment-by-visit interaction, where treatment, visit and treatment-by-visit interaction will be included as fixed factors and subject-within-site will be included as a random factor. For continuous secondary endpoints, the same statistical model will be used, with the exception that these analyses will be conducted on the original scale without log transformation. Binary secondary endpoints will be analyzed with repeated measurement models using generalized estimation equations (GEE).Safety parameters will be analyzed descriptively.

2 Abbreviations and definition of terms

AE	Adverse event
AKU	Alkaptonuria
AKUSSI	AKU Severity Score Index
APL	Apotek Produktion & Laboratorier AB
cAKUSSI	Clinical AKUSSI
CDASH	Clinical Data Acquisition Standards Harmonization
CDISC	Clinical Data Interchange Standards Consortium
CRO	Contract research organization
CRF	Case report form
DMC	Data monitoring committee
DEXA	Dual energy X-ray absorptiometry
ENT	Ear, nose and throat
EMA	European Medicines Agency
GCP	Good clinical practice
HAQ	Health Assessment Questionnaire
HGA	Homogentisic acid
HGD	Homogentisate 1,2-dioxygenase
HT-1	Hereditary tyrosinemia type 1
ICH	International Conference on Harmonisation
IEC	Independent Ethics Committee
KOOS	Knee injury and Osteoarthritis Outcome Score
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLOQ	Lower limit of quantitation
mAKUSSI	Modified AKUSSI (cAKUSSI without pigmentation items)
RLUH	Royal Liverpool University Hospital
s-HGA	Serum concentration of homogentisic acid
s-nitisinone	Serum concentration of nitisinone
s-Tyr	Serum concentration of tyrosine
SAE	Serious adverse event
SmPC	Summary of Product Characteristics
Sobi	Swedish Orphan Biovitrum
SDTM	Study Data Tabulation Model
u-HGA_{24}	24-hour urinary excretion of homogentisic acid
WORMS	Whole-Organ Magnetic Resonance Imaging Score

3 Ethics

3.1 Independent ethics committee

It is the responsibility of the investigator to obtain approval of the study protocol, possible amendments and the written patient information and informed consent form from the Independent Ethics Committee (IEC). The investigator should file all correspondence with the IEC. Copies of IEC correspondence and approvals should be forwarded to PSR Group B.V. (PSR).

3.2 Ethical conduct of the study

This study will be conducted in compliance with this protocol, the International Conference on Harmonisation (ICH) Guideline for Good Clinical Practice (GCP) [1], applicable regulatory requirements, and in accordance with the latest revision of the Ethical Principles for Medical Research Involving Human Subjects (the Declaration of Helsinki) [2].

3.3 Patient information and consent

It is the responsibility of the investigator to give each patient, prior to any study related activities, full and adequate verbal and written information regarding the objective and procedures of the study and the possible risks involved. The patients must be informed about their right to withdraw from the study at any time. The written patient information and/or consent form must not be changed without prior discussion with PSR. Before any revisions are implemented, the revised written patient information and/or consent form must be approved by the IEC.

It is the responsibility of the investigator to obtain signed informed consent from all patients prior to any study related activities. The patients should receive a copy of the written information and signed informed consent form.

4 Study administrative structure

Study sponsors:	University of Liverpool and Royal Liverpool University Hospital Trust (RLUH)
Investigators and study centers:	Professor Lakshminarayan Ranganath RLUH Prescot Street, Liverpool, Merseyside L7 8XP UK

	Professor Jozef Rovenský National Institute of Rheumatic Diseases Nabrezie Ivana Krasku 4 SK-921 01 Piešťany Slovakia Dr Jean-Baptiste Arnoux Hôpital Necker-Enfants Malades, 149, rue de Sèvres 5743 Paris Cedex 15 France.
Monitoring:	PSR Group B.V. Planetenweg 5 2132 HN Hoofddorp Netherlands
Medical Monitor:	Dr. Helena Glasová Institute of Pharmacology Slovak Medical University Limbová 14, 83303 Bratislava Slovakia
SAE reporting:	Swedish Orphan Biovitrum (Sobi) Tomtebodavägen 23A SE-112 76 Stockholm Sweden
Data management:	PSR Group B.V. Planetenweg 5 2132 HN Hoofddorp Netherlands
Statistics:	University of Liverpool Liverpool L69 3BX UK
Investigational product Market Authorization Holder:	Sobi Tomtebodavägen 23A SE-112 76 Stockholm Sweden

Investigational product manufacturing, packaging, labeling, distribution, QP release, subcontracted by Sobi to:	Apotek Produktion & Laboratorier AB (APL) Prismavägen 2 SE-141 05 Kungens Kurva Sweden
Metabolomics:	RLUH Prescot Street, Liverpool, Merseyside L7 8XP UK
Genetic mutational analysis:	Biomedical Research Center of Slovak Academy of Sciences (BMC SAV) Dubravska cesta 9, 84505 Bratislava Slovakia
Inflammatory marker analysis:	University of Siena Via Fiorentina 1, 53100 Siena Italy
Connective tissue damage analysis:	Nordic Bioscience Herlev Hovedgade 207, DK-2730 Herlev Denmark

5 Introduction

5.1 Background

5.1.1 Alkaptonuria

Alkaptonuria (AKU) is a serious, autosomal recessive, multisystem disorder [3] affecting approximately one in every 250,000 to 1 million people [4], although some countries such as Slovakia have a higher prevalence rate of around one in 19,000 [5]. The rarity of the disease makes it an ultra-orphan indication. Of 626 patients recently identified worldwide, there are 358 patients in Europe, of whom 208 are found in Slovakia [6].

Morbidity in AKU is caused by increased levels of homogentisic acid (2,5-dihydroxyphenylacetic acid, HGA) due to a deficient enzyme, homogentisate 1,2-dioxygenase (HGD) [7]. AKU patients have been shown to be homozygous or compound heterozygous for loss of function mutations in the *HGD* gene [8]. So far about 115 such mutations have been identified worldwide [9]. The absence of HGD results in patients being unable to fully metabolize the amino acid tyrosine, resulting in high plasma levels of HGA. Despite efficient and marked urinary excretion of much of the HGA formed in AKU patients [10], some of it is oxidized to a melanin-like polymeric pigment via benzoquinone acetic acid (BQA). This pigment polymer is deposited in connective tissues, particularly cartilage, a process termed

ochronosis [11], leading to severe arthritis with an early onset. There are few clinical features, aside from dark urine, until the late 20s or early 30s when progressive arthritic pain, affecting the spine and all synovial joints, large and small, begins [4, 12].

5.1.2 Current treatments for AKU

Currently, there is no pharmacological treatment available for patients with AKU and treatment options are limited to treatment of the disease sequelae as they arise, including physiotherapy, surgery and analgesia. Treatment with vitamin C to enhance HGA degradation has not proven helpful [13]. Dietary therapy restricting phenylalanine and tyrosine, which is difficult to maintain, has not shown to be effective in reducing HGA in adults, and has had no demonstrable efficacy in improving the symptoms of AKU [14].

5.1.3 Nitisinone

Nitisinone (2-[2-nitro-4-(trifluoromethyl)benzoyl] cyclohexane-1,3-dione) is a competitive inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase, which metabolizes 4-hydroxyphenlpyruvate to HGA.

Nitisinone has been shown to reduce plasma HGA levels and urinary excretion in patients with AKU and although the optimal dose had not been formally established prior to the SONIA 1 study, previous studies suggested that a dose of 2 - 4 mg per day was likely to be effective in reducing HGA levels by 90 % or more [15]. It is hypothesized that if HGA levels are reduced to, and maintained at, normal, or near normal levels in AKU patients before the onset of overt ochronosis, this might prevent the development of the debilitating clinical features of the disease. Likewise, it is hypothesized that treatment with nitisinone in patients who have already developed some degree of ochronosis, would slow down further progression of ochronosis and thereby reduce the incidence of disease related consequences.

Nitisinone also prevents the accumulation of the toxic intermediates maleylacetoacetate (MAA) and fumarylacetoacetate (FAA) in patients with hereditary tyrosinemia type 1 (HT-1). Nitisinone (Orfadin®) has had a Marketing Authorization in the European Community for treatment of HT-1 since 2005 and there is clinical experience with nitisinone from the early 1990s when the clinical study for HT-1 was initiated. Further information about Orfadin is given in the SmPC [16].

5.1.3.1 Non-clinical development of nitisinone for the treatment of AKU

Experiments in animal models and clinical studies have confirmed that nitisinone can effectively reduce HGA in AKU mouse models [17] as well as in patients [15, 18], and that ochronosis formation can be prevented by nitisinone treatment in animal models of AKU [19], as described briefly below.

The mechanism of pigmentation and how it leads to arthropathy and AKU has been investigated in human chondrocytes, an osteosarcoma cell line and in a mouse model. Cell culture models,

both of chondrocytes isolated from the joints of patients with AKU and of osteosarcoma cell lines, show that elevated HGA concentrations are the prime driver of ochronosis [20-22]. Thus osteosarcoma cell cultures in the presence of HGA accumulate ochronotic pigment in a dose-dependent fashion, whilst the pigment is absent in cells cultured in the absence of HGA [22]. Furthermore, chondrocytes from patients with AKU demonstrate significant accumulations of ochronotic pigment irrespective of whether they are taken from cartilage which has already turned black in appearance or not [22].

A mouse model for AKU has been developed [23] and these animals have a loss of function of the HGD enzyme, resulting in very high urinary HGA. A study on this mouse strain showed that a single administration of nitisinone (100 µg) could reduce the urinary output of HGA to less than 2 % of the pre-treatment level 12-24 hours after dosing [17]. With the repeated daily dosing of 25 µg of nitisinone for 4 weeks there was a continuous reduction in urinary HGA output. Despite initial difficulties in demonstrating ochronosis in this mouse model, a later study verified that these mice did indeed develop ochronosis [19]. In the latter study the animal model demonstrated elevated concentrations of HGA in plasma and the presence of ochronotic chondrocytes in several joints. As such it is a good model for the biochemistry and joint pathology of AKU. When these mice were treated with nitisinone, p-HGA concentrations were reduced by 88 % and no ochronotic pigment was observed in chondrocytes and the pericellular matrix at 67 weeks. Thus this animal model showed that treatment with nitisinone through the lifetime of the animal prevented the joint pathology subsequent to ochronosis.

These data show that this mouse model may accurately depict the HGA-induced ochronosis seen in the human pathology of AKU and further show the preventive effects of nitisinone on the ochronotic process.

5.1.4 Clinical development of nitisinone for the treatment of AKU

Prior to the European Commission Seventh Framework Programme (FP7) sponsored DevelopAKUre program (see below), nitisinone had not undergone any formal clinical development for AKU, although three published investigator-initiated studies had been completed, in which patients with AKU received varying doses of nitisinone. These are presented in Table 1.

Table 1 Completed investigator-initiated clinical studies with nitisinone in adult patients with AKU

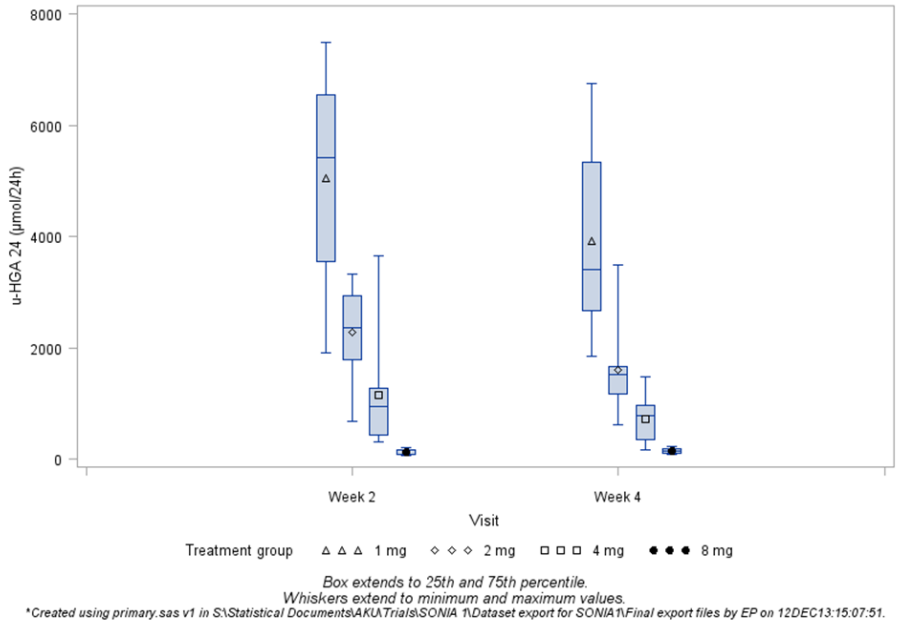
Study	Design	# patients on nitisinone	Dose of nitisinone (mg/day)	Duration of treatment	Results
Phornphutkul, Introne et al. 2002	Open, uncontrolled	2	0.7 to 2.8	9 and 10 days, respectively	Marked reduction in HGA levels
Suwannarat, O'Brien et al. 2005	Open, uncontrolled	9	0.7 to 2.1	3 months	Marked reduction in HGA levels
Introne et al. 2011	Randomized, parallel group, single blind, untreated controls	20	2	36 months	Marked reduction in HGA levels. No significant effect on clinical parameters.

In order to formally evaluate the dose-response effect of once daily nitisinone on 24-hour urinary HGA excretion after 4 weeks of treatment, an open-label, no-treatment controlled, parallel-group study was performed. That study, the SONIA 1 study, forms part of the FP7-funded DevelopAKUre program and was the basis of the dose-selection for this study (SONIA 2).

The SONIA 1 study investigated the relationship between different doses of nitisinone (1, 2, 4 and 8 mg per day) and, as primary endpoint, the 24-hour excretion of HGA (u-HGA_{24}). In addition, the serum concentrations of HGA (s-HGA) and tyrosine (s-Tyr) were evaluated. The study was performed in 40 patients with AKU; 8 patients in one of each of the nitisinone dose groups and 8 in an untreated control group. In parallel with the SONIA 1 study, u-HGA_{24} and s-HGA were also measured in 22 non-AKU subjects.

Figure 1 below shows a box plot of the u-HGA_{24} per dose over time. The u-HGA_{24} for all patients before starting treatment was highly variable, and ranged from 14,443 to 69,503 µmol (corresponding to 2,426 to 11,677 mg). For clarity of the dose-response relationship, the Week 0 values and data for untreated patients have been omitted from the figure.

Figure 1 24-hour urinary excretion (µmol/24 hours) of HGA in nitisinone-treated AKU patients at Weeks 2 and 4



Upon treatment with nitisinone, u-HGA_{24} decreased in a dose-dependent manner. Also, the inter-patient variability decreased with increasing doses. A more complete description is given in the Investigator's brochure.

Although the effect of nitisinone on HGA has been demonstrated, the 3-year study by Introne et al. [15] failed to show a statistically significant effect on clinical parameters. Several factors may have contributed to this; the dose may have been too low, the number of patients may have been too small and the treatment duration too short. Furthermore, and probably more important, the primary objective of the study was to show an effect on only one of the many manifestations of AKU; reduced range of hip motion. New tools for the assessment of disease severity in AKU have been developed and will be explored in the current study.

These tools include the AKU Severity Score Index (AKUSSI) which has been developed by the RLUH [12, 24]. The AKUSSI incorporates multiple, clinically meaningful outcomes that can be described in a single score. It includes kidney and prostate stones, aortic stenosis, bone fractures, tendon/ligament/muscle ruptures, kyphosis, scoliosis, joint replacements and all other clinical features of AKU. This score is sensitive to all morbid features of AKU and not any one feature. AKUSSI is a quantifiable, multidisciplinary assessment system, with the potential of reflecting changes in disease severity over time.

The AKUSSI, its components and the scoring system are detailed in Section 7.5.5.3.

5.2 Study rationale

5.2.1 HGA and ochronosis

As described above, nitisinone has been shown to reduce p-HGA levels and urinary excretion of HGA in patients with AKU. However, prior to the SONIA 1 study, the clinical studies had been small and had not systematically investigated the dose-response relationship between nitisinone and HGA levels. It is hypothesized that if HGA levels are reduced to and maintained at normal or near normal levels in AKU patients before the onset of overt ochronosis this might prevent the development of the debilitating clinical features of the disease.

It is thought that HGA levels are likely to be directly correlated with the development of clinical ochronosis, as evidenced by the fact that people without AKU do not develop ochronosis and by the data from in vitro and animal models discussed above. However, it is not feasible to demonstrate this in a controlled clinical trial due to the time it takes for ochronosis to develop. Therefore, a practical approach to the development of nitisinone for the treatment of AKU is to make use of HGA as a surrogate marker as far as possible, whilst still attempting to generate robust data on clinical endpoints.

The main limitation of the biochemical surrogate marker HGA is that there is currently no clinical data to support the assumption that the control of HGA levels will prevent or arrest ochronosis. From a regulatory point of view therefore, HGA may be considered as a non-qualified surrogate, even though the rationale for a link is strong and supported by non-clinical data. For this reason, the clinical relevance of a reduction in HGA needs to be supported and the SONIA 2 study is designed to obtain clinical data using a number of clinical parameters such as the AKU Severity Score Index (AKUSSI).

5.2.2 Scientific review by the EMA

The current study is part of the DevelopAKUre program, which has received funding from the FP7 project. The objective of the clinical program is to provide sufficient data for European Marketing Authorization for nitisinone in the treatment of AKU. Sobi, the Market Authorization Holder for Orfadin, has obtained Scientific Advice from the Committee for Medicinal Products for Human Use (CHMP), which has largely endorsed the development program and supported both the dose-response study (SONIA 1) and the current long-term study (SONIA 2). The CHMP recognized the importance of HGA as a surrogate biomarker in AKU and endorsed its use as the primary efficacy variable in the development program, provided it is supported by positive trends in clinical endpoints.

In accordance with the Scientific Advice from the CHMP, the present study has been designed to show effect on u-HGA as a primary endpoint. Since HGA excretion is a surrogate marker, which has not yet been qualified and no correlation between HGA levels and disease severity has so far been demonstrated, the study will also explore the effect of nitisinone on clinical endpoints and provide safety data. Twelve months of treatment is considered sufficient to confirm the

control of HGA excretion, but the slow progression of the clinical features of AKU makes it unlikely that a clear clinical benefit will be measurable at 12 months. However, if a non-statistically significant trend in clinically meaningful endpoints can be shown after 12 months (or at any point in time), the CHMP indicated in its Scientific Advice that it may consider granting a Market Authorization based on this data. A new Scientific Advice may be sought prior to a decision to file a Marketing Authorization Application.

Although there will be an interim analysis after 1 year, the study will continue up to 4 years (or until granting of a Market Authorization) in order to explore the longer-term effect on clinical endpoints and to provide longer-term safety data, subject to the conditions mentioned in Section 5.2.3 below.

5.2.3 Interim review by regulatory authorities

An interim analysis will be conducted when all patients have completed 12 months of treatment (see Section 9.3.7). This will include a complete set of efficacy and safety data up to 12 months. The report of this interim analysis will be submitted to the regulatory authorities for review. As discussed above, it is considered unlikely that a clear benefit on clinical parameters will be measurable at 12 months, but evidence of efficacy of nitisinone on biochemical parameters (e.g. HGA), together with acceptable safety, should be demonstrated in order to justify continuation of treatment up to 4 years.

5.3 Potential risks and benefits

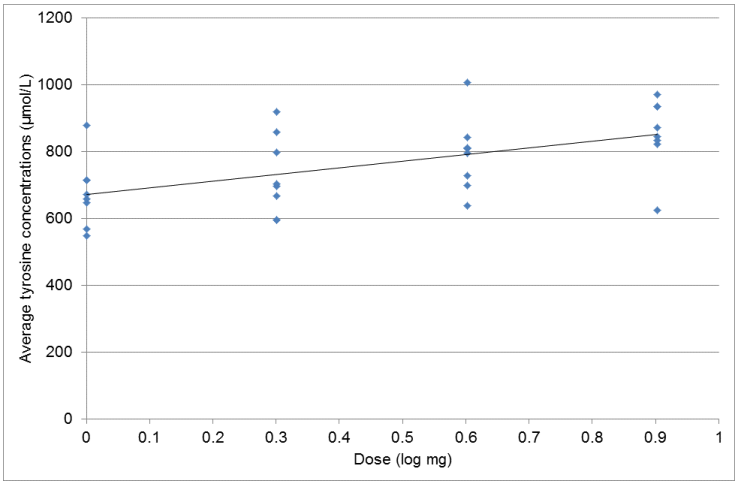
The potential risks for the study patients are considered to be minimal. Treatment with nitisinone blocks the tyrosine metabolic pathway and leads to increased plasma levels of tyrosine in a dose-dependent manner. However, tyrosine levels return to normal once nitisinone is eliminated. Elevated levels of tyrosine have been associated with corneal opacities and hyperkeratotic lesions but the effects have been shown to be reversible once nitisinone is discontinued [15].

In the SONIA 1 study, serum tyrosine concentrations (s-Tyr) were normal in all patients at baseline and increased in a dose-related manner upon treatment with nitisinone (Table 2 and Figure 2).

Table 2 Average tyrosine concentrations (µmol/L) in AKU patients at Baseline and Week 4

	0 mg N=8	1 mg N=8	2 mg N=8	4 mg N=8	8 mg N=8
Baseline	60	56	64	60	60
Week 4	61	676	729	791	855

Figure 2 Individual average serum concentrations of tyrosine vs. log dose in nitisinone-treated patients at Week 4



Even at the lowest dose of nitisinone (1 mg) there was a substantial increase in tyrosine from baseline (approximately a 10-fold increase), with higher doses producing relatively little additional increase.

Potential ocular toxicity was monitored by slit-lamp examination, the results of which are given in Table 3.

Table 3 Slit-lamp results in SONIA 1.

		Number of patients (%)				
Visit	**Result**	**Untreated (N=8)**	**1 mg (N=8)**	**2 mg (N=8)**	**4 mg (N=8)**	**8 mg (N=8)**
Week 0	Normal	5(62.5%)	7(87.5%)	6(75.0%)	5(62.5%)	6(75.0%)
	Abnormal, NCS	3(37.5%)	1(12.5%)	2(25.0%)	3(37.5%)	2(25.0%)
Week 4	Normal	6(75.0%)	7(87.5%)	7(87.5%)	6(75.0%)	5(62.5%)
	Abnormal, NCS	2(25.0%)	1(12.5%)	1(12.5%)	2(25.0%)	3(37.5%)

For the 8-mg dose group, the frequency of abnormal slit-lamp results was higher at Week 4 than at baseline. One patient had an abnormal result at Week 4 that was not reported at Baseline, but the abnormality is described as "dry eye syndrome of long term approx. 1 year", and therefore was most likely also present, but not observed or reported, at Baseline.

No patient experienced any eye related adverse events possibly related to high serum concentrations of tyrosine with concomitant abnormal slit lamp examination findings.

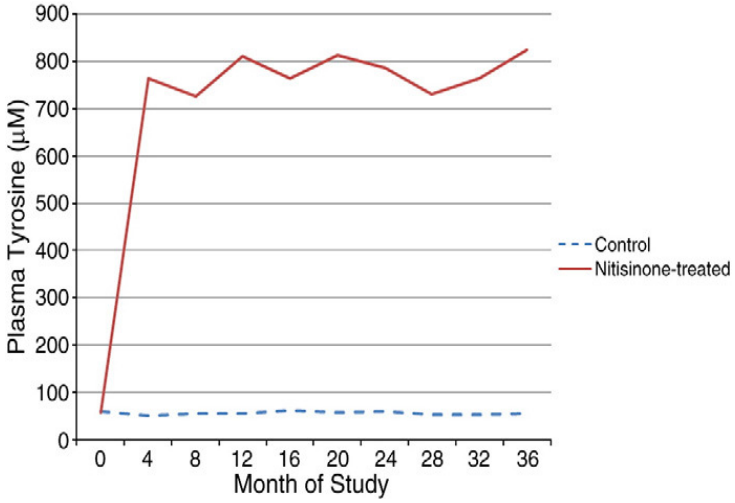
One patient in the 1-mg dose group reported foreign body sensation in eyes that started during the first week of nitisinone treatment. Slit lamp examinations of this patient at week 2 and 4 were normal and the patient continued nitisinone treatment. In addition one patient in the 4-mg dose group reported eye pain during one week. The event started one week after the last dose of nitisinone.

It is important to note that the toxic effects of nitisinone on the cornea are unlikely to manifest themselves early during nitisinone treatment. In a study of 291 patients with HT-1, eye disorders did not tend to be reported until at least 7 months after the start of nitisinone treatment [Sobi, data on file]. Therefore the lack of corneal toxicity observed in the SONIA 1 study does not preclude the potential for toxic effects at any of the doses studied.

No new safety concerns were identified in SONIA 1.

The only long-term study which has been done in AKU [15] showed a similar elevation in plasma tyrosine with nitisinone 2 mg daily, which was maintained throughout the study as shown in Figure 3 below.

Figure 3 Plasma tyrosine concentrations in control and nitisinone-treated AKU patients over time



Mean plasma tyrosine levels (µM) measured at each visit over the 36-month study period. (From Introne et al, 2011).

In that study, which included 20 patients treated with nitisinone, the elevated tyrosine levels were remarkably well tolerated; there were 8 serious adverse events (SAEs) involving 6 patients, all receiving nitisinone. A single individual developed a keratopathy classical for tyrosine toxicity approximately 6 weeks following initiation of oral nitisinone. Another patient developed elevated liver transaminases that may have been exacerbated by nitisinone use; however, her medical history was complicated and the patient was already on multiple medications associated with possible hepatotoxicity. Overall, the side effect profile of nitisinone was considered acceptable by the investigators.

For patients with HT-1, the currently licensed indication for nitisinone, the following common adverse reactions (frequency ≥1/100 to <1/10) have been reported in patients treated with Orfadin®:

Thrombocytopenia, leukopenia, granulocytopenia, conjunctivitis, corneal opacity, keratitis, photophobia, and eye pain.

It should be noted that the doses used in the treatment of HT-1 (1 to 2 mg/kg/day) are considerably higher than in the current study.

This study involves blood sampling at each visit, which are 1 year apart (with the exception of Visit 2, which takes place 3 months after visit 1 and 9 months before Visit 3). The total blood volume to be collected at each visit is 26 - 28 mL, depending on the visit.

This study involves several investigations which expose the patient to ionizing radiation. These are; DEXA scans, isotope joint (or PET/CT) scans and X-rays. The radiation exposure in patients at each visit is presented in Table 4 below.

Table 4 Ionizing radiation dose per patient

Assessment	Radiation dose per visit	Visits at which performed	Total radiation dose
Dual Energy X-ray Absorptiometry (DEXA) scan of hip	0.001	0, 12, 24, 36, and 48 months	0.005 mSv
Technetium labeled methylene diphosphonate (Tc^{99m} MDP) scan of joints and spine	3	0, 12, 24, 36, and 48 months	15 mSv
F^{18}-Fluoride PET/CT scan of joints and spine(as alternative to Tc^{99m} MDP)	4.8 for F^{18}-Fluoride PET 3.7 for low-dose CT	0, 12, 24, 36, and 48 months	42.5 mSv (including low-dose CT 3.7 mSv per exam)
X-ray spine and target joint	0.7 x 2	0, 12, 24, 36, and 48 months	7 mSv
		Total	**22.005 or 49.505 mSv**

If the patient has F^{18}-Fluoride scan + X-ray spine, the dose of radiation is approximately 50 mSv.

Since 1 mSv gives an additional theoretical cancer risk of 1 in 20,000, therefore 50 mSv gives an additional cancer risk of 1 in 400.

If the patient has Tc^{99m} MDP scan + X-ray spine, the dose of radiation is approximately 22 mSv.

Since 1 mSv gives an additional cancer risk of 1 in 20,000, therefore 22 mSv gives an additional cancer risk of 1 in 909.

These figures have to be considered in the context of lifetime risk of developing cancer, which is 1 in 3 for all ages. Since these scans have the potential to positively influence patient management, the additional risk of developing cancer can be considered small and justifiable. In addition, this radiation risk should also be interpreted in the context of any natural reduction in life expectancy in patients with alkaptonuria (footnote 1).

[1] Personal communication from Professor Sobhan Vinjamuri (an International Expert to the Nuclear Medicine Section, Division of Human Health of IAEA).

Potential benefits of participation:

Although nitisinone treatment may be expected to prevent ochronosis in patients who are treated before the development of clinical manifestations, an assumption which is strongly supported by animal model data, there is currently no evidence to support the hypothesis that treatment of patients with existing ochronosis may slow disease progression. The current study will therefore test this hypothesis as a secondary objective and, due to the long treatment period, patients randomized to nitisinone may show a slower progression in the deterioration of clinical parameters compared to the control (no active treatment group).

Patients randomized to the control group will also benefit from participation in the study, since they will receive state-of-the-art specialist medical screening, investigations and control which is often not accessible for patients with very rare diseases. Due to the lack of any evidence that nitisinone treatment can be beneficial in patients with established ochronosis, together with the small but real risks of long-term treatment with nitisinone, the inclusion of a no active treatment group for a 4-year period is considered to be scientifically and ethically justified.

6 Study objectives and endpoints

6.1 Primary objective

To demonstrate that nitisinone is superior compared to no treatment in reducing 24-hour urinary homogentisic acid excretion in patients with alkaptonuria after 12 months.

6.1.1 Primary endpoint

u-HGA_{24} after 12 months.

6.2 Secondary objectives

- To demonstrate the effect of nitisinone on control of u-HGA_{24} after 3, 24, 36 and 48 months.
- To demonstrate the effect of nitisinone on control of serum HGA concentration (s-HGA) in patients with AKU after 3, 12, 24, 36 and 48 months of treatment.
- To demonstrate the effect of nitisinone on pre-defined clinical parameters.
- To assess the association between u-HGA_{24} and change in clinical parameters.
- To assess predose serum concentrations of nitisinone after 3, 12, 24, 36 and 48 months of treatment.
- To assess the association between u-HGA_{24} and predose serum concentrations of nitisinone.

- To assess the effect of nitisinone on pre-defined measures of health and functional status, as assessed by SF-36, the Health Assessment Questionnaire (HAQ) and Knee injury and Osteoarthritis Outcome Score (KOOS).
- To assess the safety of long-term treatment with nitisinone in patients with AKU.

6.2.1 Secondary endpoints

- u-HGA_{24} after 3, 24, 36 and 48 months.
- Occurrence of achieved target level (< 300 µmol) of u-HGA_{24} at 3, 12, 24, 36 and 48 months.
- Predose s-HGA at 3, 12, 24, 36 and 48 months.
- Change from Baseline in Clinical AKUSSI (cAKUSSI) scores at 12, 24, 36 and 48 months.
- Change from Baseline in Modified AKUSSI scores at 12, 24, 36 and 48 months.
- Change from Baseline in individual cAKUSSI items at 12, 24, 36 and 48 months.
-
- Change from Baseline in ear cartilage pigmentation at 48 months.
- Change from Baseline in range of joint and spine motion at 12, 24, 36 and 48 months.
- Change from Baseline in other pre-defined rheumatology assessments at 12, 24, 36 and 48 months.
- Change from Baseline in quality of life (QoL) measured by SF-36 at 12, 24, 36 and 48 months.
- Change from Baseline in health assessment measured (including pain scores) by HAQ at 12, 24, 36 and 48 months.
- Change from Baseline in physical function as measured by KOOS index at 12, 24, 36 and 48 months.
- Predose serum nitisinone at 3, 12, 24, 36 and 48 months.
- Adverse events, serum concentration of tyrosine (s-Tyr), clinical chemistry and hematology, vital signs, electrocardiogram (ECG) and corneal eye assessments.

6.3 Exploratory objectives

- To explore the effect of nitisinone on inflammatory biomarkers, and biomarkers of bone, cartilage and cardiovascular damage.
- To explore the effect of nitisinone on metabolites of tyrosine (other than HGA).
- To explore metabolic pathways in AKU patients (metabolomics).

- To explore the effect of nitisinone on spine and joint disease as assessed by magnetic resonance imaging and knee radiographs.
- To explore phenotype/genotype correlations.
- To explore the use of digital image analysis of photographs, X-rays and scans as a measure of disease progression of AKU.

6.3.1 Exploratory endpoints

- Biomarkers of inflammation at Baseline.
- Biomarkers of bone, cartilage and cardiovascular damage at Baseline.
- Biomarkers of inflammation and of bone, cartilage or cardiovascular damage which were elevated at Baseline, at 3, 12, 24, 36 and 48 months.
- Tyrosine metabolites other than HGA.
- Metabolomics.
- Measures of spine and joint disease
- AKU genotype.
- Digital image analyses of photographs, X-rays and scans.

The results of the exploratory assessments will be presented in separate reports and not included in the Clinical Study Reports.

7 Investigational plan

7.1 Overall study design and plan

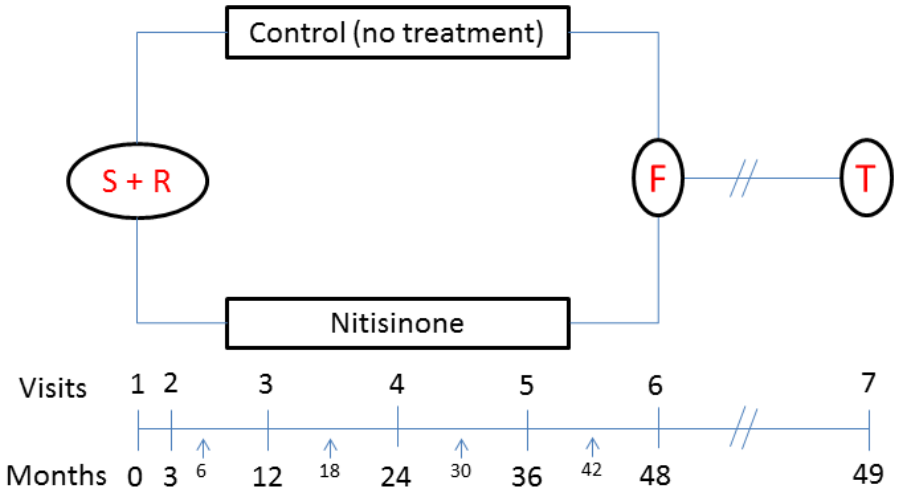
This study is a randomized, open-label, evaluator-blinded, parallel-group study with a no-treatment control group. The study duration is 48 months with a 12-month interim analysis. The purpose of the interim analysis is to evaluate if data support the submission of a Marketing Authorization Application in Europe (see Section 5.2.2). It will also be used to assess the efficacy of nitisinone on biochemical markers and safety in order to justify continuation of treatment up to 4 years (see Section 5.2.3).

Patients will be randomized to receive either nitisinone or no treatment (control).

One hundred and forty (140) patients will be randomized, equally distributed amongst the groups (70 patients per group).

The study design is summarized in Figure 4.

Figure 4 Overall design of the SONIA 2 study



S + R = Screening, Baseline and Randomisation Visit
F = Final treatment Visit
T = Telephone follow-up Visit
↑ 18 = Interim safety questionnaires

In addition to the site visits, a questionnaire to collect safety information will be completed by the patient at 6, 18, 30 and 42 months (i.e., at the mid-point between the annual visits).

This study is part of the DevelopAKUre program, which has received funding from the European Commission 7th Framework Program (FP7). The DevelopAKUre Advisory Board, which consists of six members who do not participate in the project itself but who are known experts in the field of AKU, will have the role as the Steering Committee for the present study.

7.2 Discussion of study design

This study is designed based on feedback from the CHMP at the Scientific Advice meeting, as described in Section 5.2. Also discussed with the CHMP was the fact that it is not feasible to double-blind a study with nitisinone in AKU; one of the signs of AKU is urine which turns black on standing, so patients can easily find out if they are receiving active drug or not. Assessments which require direct contact between investigator and patient cannot be reliably blinded, as there is no way to prevent the patient from unblinding the investigator. It was agreed to make the study evaluator-blinded, such that assessments which do not require direct contact between the evaluator and the patient will be blinded.

For procedures which generate a numerical read-out from the equipment, the number(s) will be entered directly into the Viedoc™ system (see Section 10.2 for a description of the system). For

procedures which generate an image, the image(s) will be sent for central evaluation by a blinded evaluator, and the results of the evaluation entered into Viedoc.

Access to the study database will be restricted to the data manager and the statistical programmer. These will not have access to the randomization code until after database lock, following completion of the 1-year time-point. After that, these individuals can no longer be blinded, however the clinical evaluators will remain blinded after the 1-year analysis.

Since this study uses objective assessments of efficacy for the primary endpoint (HGA levels), the lack of double-blind is unlikely to introduce bias in the primary endpoint or those which are based on evaluator-blinded assessments. However, it is recognized that reporting of subjective assessments may introduce bias for some of the secondary endpoints, such as pain and Quality of Life assessments and reporting of adverse events.

7.3 Selection of study population

7.3.1 Inclusion criteria

A patient must fulfill the following criteria in order to be included in the study:

1. Diagnosis of AKU.
2. Any clinical manifestations of AKU, such as clinical ochronosis or chronic back / joint pain.
3. Age ≥25 years.
4. Willing and able to visit the investigational site for study visits.
5. Signed written informed consent given.

7.3.2 Exclusion criteria

The presence of any of the following will exclude a patient from inclusion in the study:

1. Treatment with nitisinone within 3 months of randomization.
2. Participation in another clinical study within 3 months of randomization.
3. Known allergy to nitisinone or any of the constituents of the investigational product.
4. Female patient of child-bearing potential not using a reliable method of contraception.
5. Currently pregnant or lactating.
6. Current malignancy.
7. Uncontrolled hypertension (blood pressure greater than 180 mmHg systolic or greater than 95 mmHg diastolic).
8. Unstable cardiovascular disease.

9. Serum potassium < 3.0 mmol/L.
10. eGFR < 60 mL/min (footnote 2).
11. ALT > 3 x upper limit of normal.
12. Hemoglobin < 10.0 g/dL.
13. Platelets < 100 x 10^9/L.
14. Total white blood count < 3.0 x 10^9/L or neutrophil count < 1.5 x 10^9/L.
15. History of alcohol or drug abuse.
16. Psychiatric or somatic illness that interferes with compliance or communication with health care personnel.
17. Foreseeable inability to cooperate with given instructions or study procedures.
18. Any other medical condition which in the opinion of the investigator makes the patient unsuitable for inclusion.

7.3.3 Withdrawal of patients from treatment or study

A patient should be withdrawn from treatment if the patient:

- Develops ocular signs or symptoms, or
- Develops a skin rash (hyperkeratotic lesions)

which are judged by the investigator to be related to elevated tyrosine (see Sections 7.3.4 and 7.5.6.1.6).

A patient should also be withdrawn from the study if the patient:

- Becomes pregnant.
- Develops allergy to nitisinone or any of the constituents of the investigational product.
- ALT increases to > 3 x upper limit of normal.
- Platelets decrease to < 100 x 10^9/L.
- Total white blood count decreases to < 2.5 x 10^9/L or neutrophils < 1.0 x 10^9/L.

Furthermore, a patient should be withdrawn from the study if, in the opinion of the investigator, it is medically necessary, or if it is the wish of the patient.

[2] Glomerular filtration rate estimated using the MDRD method. The calculation is performed in the eCRF upon entering the serum creatinine value

If a patient is withdrawn from the study, the date of last Investigational Medicinal Product (IMP) dose and the date and reason for withdrawal should be clearly described in the relevant sections of the case report form (CRF).

If a patient is withdrawn from the study because of an adverse event (AE), the reason for treatment withdrawal should always be stated as 'adverse event' irrespective of whether this was the investigator's or the patient's decision.

The withdrawn patient should, whenever possible, irrespective of the reason for withdrawal, as soon as possible be examined. Relevant samples should be obtained and all relevant assessments should be completed, preferably according to the schedule for Visit 6. The CRF should be completed as far as possible.

7.3.4 Replacement of withdrawn patients

Withdrawn patients will not be replaced. Patients withdrawn due to an adverse event must not re-enter the study.

Patients temporarily withdrawn from treatment due to suspected tyrosine toxicity may continue in the study on a lower dose of nitisinone (2 mg) at the discretion of the investigator and only once all signs and symptoms of tyrosine toxicity have resolved. Patients who are temporarily withdrawn due to a suspected, but not later confirmed, tyrosine toxicity, may continue in the study on the 10-mg dose. In both cases, dates for temporary withdrawal of nitisinone, the date when treatment is reinitiated, and the dose used after the pause, will be recorded in the CRF.

7.3.5 Specific restrictions/requirements on patients

- Female patients should use a reliable method of contraception during the study and for one month after the study.
- Patients will be advised to limit their protein intake in order to keep tyrosine levels as low as possible without introducing specific diets. The importance of controlling protein and phenylalanine intake will be emphasized and a list of foods particularly high in tyrosine & phenylalanine will be provided.
- Patients will be recommended to avoid using contact lenses during the study.

7.4 Treatments

7.4.1 Treatments administered

For this study, nitisinone (Orfadin®) capsules, currently registered for the treatment of HT-1, will be used. The dose and capsule strength to be used was determined after analysis of the SONIA 1 study (see Section 7.4.4).

Table 5 Treatments administered

Investigational product	Dosage form	Route	Daily dose	Dosage regimen
None	Not applicable	Not applicable	Not applicable	Not applicable
Nitisinone (Orfadin®)	Capsules	Oral	10 mg	Once daily

7.4.2 Identity of investigational medicinal products

Orfadin® is available as capsules containing 2, 5 or 10 mg of nitisinone. The dose and capsule strength to be used as IMP in this study (10 mg) was determined after analysis of the SONIA 1 study (see Section 7.4.4). A dose of 2 mg may be used in case a patient develops tyrosine-related adverse events.

Sobi is the license holder of Orfadin for the treatment of HT-1, and supplier of IMP for this study. The IMP will be manufactured, packaged and labeled by Apotek Produktion & Laboratorier (APL), Kungens Kurva, Sweden. The IMP will be labeled in accordance with regulatory requirements (Article 26 of Annex 13 of the EU Good Manufacturing Practice) and will include the following information, as a minimum:

- Identity of the study (SONIA 2).
- Identity, strength, batch number, expiry date and storage requirements.
- Principal investigator's name and phone number.
- "For clinical trial use only".
- "Keep out of reach of children".

The IMP should be stored refrigerated (2 to 8°C) at the study site, and at room temperature not exceeding 25°C during use.

Possible deficiencies related to the handling and quality of the IMP should be reported to the study monitor and also directly to complaints@sobi.com.

7.4.3 Method of assigning patients to a treatment group

Patients will be randomly assigned to one of the two groups in a 1:1 ratio. The randomization will be stratified by study center and age (≤ 55 years and > 55 years), since there is evidence that the rate of increase in AKUSSI increases after the age of (approximately) 55.

The study statistician will create a program to randomly assign the patients to the two treatment groups using the SAS System. The randomization will be carried out by using randomly permuted blocks within each study center and age stratum. The block size used in the randomization will be kept blinded during the conduct of the study. An independent randomization expert at the University of Liverpool will execute the randomization program.

The documentation required to recreate the randomization (i.e., code list and seed number) will be kept stored by the randomization expert.

The randomization will be centrally implemented in Viedoc. See Section 10.2.

7.4.4 Selection of dose

The choice of dose to be used in the present study is based on current knowledge regarding the HGA-lowering effect of nitisinone and the request from the European Medicines Agency (EMA) (Scientific Advice 15 November 2012) to lower HGA to normal, physiological levels (thought to be u-HGA_{24} < 100 mg/day, as indicated in the literature [18]). A study in subjects without AKU carried out at the Royal Liverpool University Hospital found that serum concentrations of HGA (s-HGA) could not be quantified, or even detected, in any of the 22 non-AKU subjects, since all concentrations were below the lower limit of quantification (LLOQ) of 3.1 µmol/L. The mean u-HGA excretion in normal subjects could not be calculated due to a majority (15/22) of HGA concentrations being below the LLOQ of 1 µmol/L. The SONIA 1 study investigated the effect of 1, 2, 4 and 8 mg nitisinone per day in a formal dose-response study. The goal of normalizing u-HGA_{24} was not quite reached, not even with the highest dose. However, the 8-mg dose resulted in an average reduction of u-HGA_{24} of 99.4 % compared to baseline. Considering that a complete normalization of u-HGA_{24} would mean a reduction by 99.99 %, and that a reduction by 99.4 % is achieved with the 8-mg dose, this dose is considered sufficiently high for use in SONIA 2, despite not completely normalizing the HGA excretion.

It is not possible to be certain what dose would be needed for a complete normalization of u-HGA. Adult patients with HT-1, a disease that requires 100 % enzyme inhibition due to the extremely toxic tyrosine metabolites formed, use daily doses of 50 mg or more [Sobi, data on file]. It is possible that a dose on that level would be necessary for a suppression of the last 0.6 % of u-HGA_{24}, since the dose-response curve is very flat toward the end of the studied dose interval.

Since AKU is a non-fatal disease (unlike HT-1) and tyrosine metabolites in AKU are not so toxic, the risk-benefit ratio of nitisinone treatment is different to that for HT-1. The nitisinone dose should be kept as low as possible, whilst achieving sufficient efficacy. From the results of the SONIA1 study, the most efficacious dose, with the least inter-subject variability was 8 mg. This dose did not produce any safety concerns in the SONIA 1 study. The closest available capsule strength is 10 mg and therefore the SONIA 2 will be performed using one 10 mg capsule daily.

The dosing frequency is based on the half-life of nitisinone in adult subjects of around 50 hours [25], which makes the drug suitable for once daily dosing.

7.4.5 Selection and timing of doses for each patient

Nitisinone will be taken in the morning.

7.4.6 Blinding

This is an open-label study. However, assessments will be blinded whenever possible (see Section 7.2).

7.4.7 Prior and concomitant therapy

There will be no restrictions for the patients regarding concomitant medication, but all therapy on-going at inclusion, and all changes made during the study, must be recorded in the CRF.

Patients must not have used nitisinone within 3 months prior to Visit 1. No other IMP may be used concomitantly with the IMP in this study, or within 3 months prior to Visit 1.

7.4.8 Treatment compliance

Product accountability records will be kept. The pharmacy and investigator must maintain accurate records demonstrating date and amount of IMP received, to whom and by whom administered or dispensed (patient-by-patient accounting), and accounts of returned IMP and any IMP accidentally or deliberately destroyed. All unused IMP will be counted. At the end of the study, any remaining IMP will be destroyed locally. A certificate of destruction must be issued.

7.5 Efficacy, safety, pharmacokinetic, and genetic assessments

7.5.1 Study schedule

The Schedule of Events for the study is provided in Section 7.5.1.1 below.

Patients in the no-treatment control arm will undergo all the study procedures and investigations except they will not take any study medication.

7.5.1.1 Schedule of events

Table 6 Schedule of events

	Visit 1 Month 0	Visit 2 Month 3	Visit 3 Month 12	Visit 4 Month 24	Visit 5 Month 36	Visit 6 Month 48	Visit 7 Month 49[1]
Informed consent	X						
Demographics	X						
Medical history	X						
Physical examination[2]	X	X	X	X	X	X	
Inclusion/exclusion criteria	X						
Randomization	X						
Vital signs	X	X	X	X	X	X	
12-lead ECG	X	X	X	X	X	X	
Hematology	X	X	X	X	X	X	
Clinical chemistry	X	X	X	X	X	X	
Pregnancy test[3]	X		X	X	X	X	
Corneal eye assessment	X	X	X	X	X	X	
Treatment with nitisinone (treatment group only)	←	—	—	—	—	→	
24-hour urine for HGA	X	X	X	X	X	X	
s-HGA, s-Tyr, s-nitisinone	X	X	X	X	X	X	
AKUSSI assessments	X		X	X	X	X	
Eye and ear pigmentation	X		X	X	X	X	
Prostate and renal stones	X		X	X	X	X	
Osteopenia of the hip	X		X	X	X	X	
Adult fractures	X		X	X	X	X	
Tendon, ligament and muscle ruptures	X		X	X	X	X	
Aortic stenosis and sclerosis	X		X	X	X	X	
Hearing impairment	X		X	X	X	X	
Dark eardrum	X		X	X	X	X	
Joint and spine pain	X		X	X	X	X	
Osteoarticular disease of joints and spine	X		X	X	X	X	
Kyphosis and scoliosis	X		X	X	X	X	
Arthroscopies and joint replacements	X		X	X	X	X	

Continued on next page

	Visit 1 Month 0	Visit 2 Month 3	Visit 3 Month 12	Visit 4 Month 24	Visit 5 Month 36	Visit 6 Month 48	Visit 7 Month 49[1]
Magnetic resonance imaging of spine and target joint and knee radiographs	X		X			X	
Ear cartilage biopsy[4]	X					X	
Range of motion of spine and joints and related rheumatology assessments	X		X	X	X	X	
Range of motion	X		X	X	X	X	
Modified Schober test	X		X	X	X	X	
20-meter walk test	X		X	X	X	X	
6-minute walk test	X		X	X	X	X	
SF-36 Health Survey	X		X	X	X	X	
Knee injury and Osteoarthritis Outcome Score (KOOS)	X		X	X	X	X	
Health Assessment Questionnaire (HAQ)	X		X	X	X	X	
Urine metabolomics	X	X	X	X	X	X	
Plasma metabolomics	X	X	X	X	X	X	
Urine biomarkers	X	X	X	X	X	X	
Serum biomarkers	X	X	X	X	X	X	
Genetic profile	X						
Dispense nitisinone (treatment group only)	X	X	X	X	X		
Drug accountability (treatment group only)		X	X	X	X	X	
Prior and concomitant medication	X	X	X	X	X	X	X
Adverse Events[5]	X	X	X	X	X	X	X

1) Telephone contact only.
2) Including weight and height.
3) Women of childbearing potential.
4) Optional: patients may participate in the study even if they decline ear biopsy.
5) All SAEs reported to the follow-up visit (Visit 7), or at least 28 days after Visit 6, and thereafter only SAEs judged to be related to the IMP.

In addition to the site visits, an interim safety questionnaire will be completed at Months 6, 18, 30 and 42 by the patient to collect safety information specifically related to tyrosine toxicity. Telephone contact with the patient will be made if indicated by the response to these questions (see Section 7.5.6.7).

7.5.1.2 Visit 1 (Screening and Baseline visit): Month 0

The schedule for this visit is described in detail in Table 7.

Table 7. Schedule of events at Visit 1

Day 1 Screening + Baseline
Patient is admitted to the clinic in the morning.
Obtain written, informed consent prior to any study-specific procedures being carried out.
Record demographics (including date of birth, sex and race).
Check inclusion / exclusion criteria, including the following:
Take complete medical history, including history of AKU.
Record prior and concomitant medication, including the names of all current prescription and over the counter (OTC) medications, vitamins and supplements including start date, dosage, frequency, route, and indication for usage within 30 days of consent.
Perform physical examination, including the following: head and neck, eyes, ENT (ears, nose and throat), heart, lung, chest, abdomen, skin, musculoskeletal, neurological, endocrine and lymphatic system, and weight and height.
Record vital signs (pulse rate, blood pressure and body temperature).
Record 12-lead ECG.
Collect blood samples for hematology and clinical chemistry, if no recent results (<1 month) are available.
Perform pregnancy test (premenopausal unsterilized female patients).
If all inclusion criteria and none of exclusion criteria are met, the patient is randomized. Pharmacy prepares bottle with nitisinone capsules.
Perform all assessments required to generate AKUSSI (Eye and ear pigmentation, Prostate and renal stones, Osteopenia of the hip, Adult fractures, Tendon, ligament and muscle ruptures, Aortic stenosis and sclerosis, Hearing impairment, Dark eardrum, Joint and spine pain, Osteoarticular disease of joints and spine, Kyphosis and scoliosis, Arthroscopies and joint replacements). Perform other clinical assessments which are not part of the AKUSSI (Magnetic resonance imaging of spine and target joints, Knee radiographs, Ear cartilage biopsy, Range of motion of spine and joints and related rheumatology assessments, SF-36 Health Survey, Knee injury and Osteoarthritis Outcome Score [KOOS], Health Assessment Questionnaire [HAQ]). Perform corneal eye assessment. Assessments may continue on Days 2-3 as required, but must not interfere with sample collection.

Continued on next page

Days 2-3 Baseline, continued	
Time (hours)	
Wake up	Collect first urine voided for biomarkers and metabolomics.
Approx. -1	Collect fasting serum samples for tyrosine, HGA, nitisinone, and biomarkers, plasma for metabolomics and blood for genetics.
Approx.-0.5	Eat breakfast.
t=0	Empty bladder. Urine discarded.
> 0 to < 24	Collect all urine for 24-hour HGA.
	Continue with assessments not completed on Day 1.
24	Empty bladder. This last portion is included in the 24-hour urine.
24 +	After all assessments are completed, administer first dose of nitisinone and dispense nitisinone for the next 3 months (if applicable).
	Instruct patient on use of (electronic) questionnaire and diary. Patient is discharged.

Procedures may continue to a fourth day if required by logistics. This will not affect the sequence or extent of the procedures.

Patients will remain as in-patients at the hospital for the two or three nights of Visit 1.

7.5.1.3 Visit 2: Month 3

This visit should take place 3 months after intake of the first dose of study medication (+/- 2 weeks). The timing of the procedures are described in detail in Table 8.

Table 8. Schedule of events at Visit 2

Day 1
If feasible, the patient should arrive fasting. On arrival at hospital, empty bladder. This urine, provided it is collected from a fasting patient, is used for biomarkers and metabolomics. Note time when bladder is emptied, i.e., start of 24-hour collection period. (The nitisinone dose should be taken at the normal time, irrespective of when the patient arrives at the clinic, and the time of intake recorded in the CRF.)
Collect all urine during the following 24 hours.
Record concomitant medications and adverse events since the previous visit.
Perform physical examination (as per Visit 1), 12-lead ECG and record vital signs.
Perform corneal eye assessment for signs of tyrosine toxicity.
Day 2
Collect fasting predose serum samples for tyrosine, HGA, nitisinone and biomarkers, plasma for metabolomics and blood samples for hematology and clinical chemistry (patient remains fasting and does not take nitisinone dose until after sample collection).
After sample collection, administer nitisinone and breakfast (in any order).
Empty bladder exactly 24 hours after start of urine collection period. This portion is included in the 24-hour urine.
Continue with assessments not completed on Day 1.
Register new adverse events.
Perform drug accountability.
Provide patient with initial supply of nitisinone and ensure that further supply until next visit is arranged (if applicable).
Patient is discharged.

Procedures may continue to a third day if required by logistics. This will not affect the sequence or extent of the procedures.

If urine collected upon arrival was not collected in the fasted state, a urine sample for biomarkers will need to be collected before breakfast on Day 3.

Patients will remain as in-patients at the hospital for the one or two nights of Visit 2.

7.5.1.4 Visit 3: Month 12

This visit should take place 12 months after intake of the first dose of study medication (+/- 3 weeks). The timing of the procedures are described in detail in Table 9.

Table 9. Schedule of events at Visit 3

Day 1
If feasible, the patient should arrive fasting. On arrival at hospital, empty bladder. This urine, provided it is collected from a fasting patient, is used for biomarkers and metabolomics. Note time when bladder is emptied, i.e., start of 24-hour collection period. (The nitisinone dose should be taken at the normal time, irrespective of when the patient arrives at the clinic, and the time of intake recorded in the CRF.)
Collect all urine during the following 24 hours.
Record concomitant medications and adverse events since the previous visit.
Perform physical examination (as per Visit 1), 12-lead ECG and record vital signs.
Perform corneal eye assessment for signs of tyrosine toxicity.
Perform pregnancy test (premenopausal unsterilized female patients).
Perform all assessments required to generate AKUSSI (Eye and ear pigmentation, Prostate and renal stones, Osteopenia of the hip, Adult fractures, Tendon, ligament and muscle ruptures, Aortic stenosis and sclerosis, Hearing impairment, Dark eardrum, Joint and spine pain, Osteoarticular disease of joints and spine, Kyphosis and scoliosis, Arthroscopies and joint replacements). Perform other clinical assessments which are not part of the AKUSSI (Magnetic resonance imaging of spine and target joints, Knee radiographs, Range of motion of spine and joints and related rheumatology assessments, SF-36 Health Survey, Knee injury and Osteoarthritis Outcome Score [KOOS], Health Assessment Questionnaire [HAQ]). Note: Ear cartilage biopsy is not performed at this visit. Assessments may continue on Days 2-3 as required, but must not interfere with sample collection.
Days 2-3
On Day 2, collect fasting predose serum samples for tyrosine, HGA, nitisinone and biomarkers, plasma for metabolomics and blood samples for hematology and clinical chemistry (patient remains fasting and does not take nitisinone dose until after sample collection).
After sample collection, administer nitisinone and breakfast (in any order).
Empty bladder exactly 24 after start of urine collection period. This portion is included in the 24-hour urine.
Continue (also on Day 3) with assessments not completed on Day 1.
If urine collected upon arrival was not collected in the fasted state, a urine sample for biomarkers is collected before breakfast on Day 3.
Register new adverse events.
Perform drug accountability.
Provide patient with initial supply of nitisinone and ensure that further supply until next visit is arranged (if applicable).
Patient is discharged.

Procedures may continue to a fourth day if required by logistics. This will not affect the sequence or extent of the procedures.

Patients will remain as in-patients at the hospital for the two or three nights of Visit 3.

7.5.1.5 Visit 4: Month 24

This visit should take place 24 months after intake of the first dose of study medication (+/- 3 weeks). The timing of the procedures are described in detail in Table 10.

Table 10. Schedule of events at Visit 4

Day 1
If feasible, the patient should arrive fasting. On arrival at hospital, empty bladder. This urine, provided it is collected from a fasting patient, is used for biomarkers and metabolomics. Note time when bladder is emptied, i.e., start of 24-hour collection period. (The nitisinone dose should be taken at the normal time, irrespective of when the patient arrives at the clinic, and the time of intake recorded in the CRF.)
Collect all urine during the following 24 hours.
Record concomitant medications and adverse events since the previous visit.
Perform physical examination (as per Visit 1) 12-lead ECG and record vital signs.
Perform corneal eye assessment for signs of tyrosine toxicity.
Perform pregnancy test (premenopausal unsterilized female patients).
Perform all assessments required to generate AKUSSI (Eye and ear pigmentation, Prostate and renal stones, Osteopenia of the hip, Adult fractures, Tendon, ligament and muscle ruptures, Aortic stenosis and sclerosis, Hearing impairment, Dark eardrum, Joint and spine pain, Osteoarticular disease of joints and spine, Kyphosis and scoliosis, Arthroscopies and joint replacements). Perform other clinical assessments which are not part of the AKUSSI (Range of motion of spine and joints and related rheumatology assessments, SF-36 Health Survey, Knee injury and Osteoarthritis Outcome Score [KOOS], Health Assessment Questionnaire [HAQ]). Note: MRI, Knee radiographs, and Ear cartilage biopsy are not performed at this visit. Assessments may continue on Days 2-3 as required, but must not interfere with sample collection.
Days 2-3
On Day 2, collect fasting predose serum samples for tyrosine, HGA, nitisinone and biomarkers, plasma for metabolomics and blood samples for hematology and clinical chemistry (patient remains fasting and does not take nitisinone dose until after sample collection).
After sample collection, administer nitisinone and breakfast (in any order).
Empty bladder exactly 24 after start of urine collection period. This portion is included in the 24-hour urine.
Continue (also on Day 3) with assessments not completed on Day 1.
If urine collected upon arrival was not collected in the fasted state, a urine sample for biomarkers is collected before breakfast on Day 3.
Register new adverse events.
Perform drug accountability.
Provide patient with initial supply of nitisinone and ensure that further supply until next visit is arranged (if applicable).
Patient is discharged.

Procedures may continue to a fourth day if required by logistics. This will not affect the sequence or extent of the procedures.

Patients will remain as in-patients at the hospital for the two or three nights of Visit 4.

7.5.1.6 Visit 5: Month 36

This visit should take place 36 months after intake of the first dose of study medication (+/- 3 weeks). The timing of the procedures are described in detail in Table 11.

Table 11. Schedule of events at Visit 5

Day 1
If feasible, the patient should arrive fasting. On arrival at hospital, empty bladder. This urine, provided it is collected from a fasting patient, is used for biomarkers and metabolomics. Note time when bladder is emptied, i.e., start of 24-hour collection period. (The nitisinone dose should be taken at the normal time, irrespective of when the patient arrives at the clinic, and the time of intake recorded in the CRF.)
Collect all urine during the following 24 hours.
Record concomitant medications and adverse events since the previous visit.
Perform physical examination (as per Visit 1), 12-lead ECG and record vital signs.
Perform corneal eye assessment for signs of tyrosine toxicity.
Perform pregnancy test (premenopausal unsterilized female patients).
Perform all assessments required to generate AKUSSI (Eye and ear pigmentation, Prostate and renal stones, Osteopenia of the hip, Adult fractures, Tendon, ligament and muscle ruptures, Aortic stenosis and sclerosis, Hearing impairment, Dark eardrum, Joint and spine pain, Osteoarticular disease of joints and spine, Kyphosis and scoliosis, Arthroscopies and joint replacements). Perform other clinical assessments which are not part of the AKUSSI (Range of motion of spine and joints and related rheumatology assessments, SF-36 Health Survey, Knee injury and Osteoarthritis Outcome Score [KOOS], Health Assessment Questionnaire [HAQ]). Note: MRI, Knee radiographs, and Ear cartilage biopsy are not performed at this visit. Assessments may continue on Days 2-3 as required, but must not interfere with sample collection.
Days 2-3
On Day 2, collect fasting predose serum samples for tyrosine, HGA, nitisinone and biomarkers, plasma for metabolomics and blood samples for hematology and clinical chemistry (patient remains fasting and does not take nitisinone dose until after sample collection).
After sample collection, administer nitisinone and breakfast (in any order).
Empty bladder exactly 24 after start of urine collection period. This portion is included in the 24-hour urine.
Continue (also on Day 3) with assessments not completed on Day 1.
If urine collected upon arrival was not collected in the fasted state, a urine sample for biomarkers is collected before breakfast on Day 3.
Register new adverse events.
Perform drug accountability.
Provide patient with initial supply of nitisinone and ensure that further supply until next visit is arranged (if applicable).
Patient is discharged.

Procedures may continue to a fourth day if required by logistics. This will not affect the sequence or extent of the procedures.

Patients will remain as in-patients at the hospital for the two or three nights of Visit 5.

7.5.1.7 Visit 6 (End-of-treatment visit): Month 48

This visit should take place 48 months after intake of the first dose of study medication (+/- 4 weeks). The timing of the procedures are described in detail in Table 12.

Table 12. Schedule of events at Visit 6

Day 1
If feasible, the patient should arrive fasting. On arrival at hospital, empty bladder. This urine, provided it is collected from a fasting patient, is used for biomarkers and metabolomics. Note time when bladder is emptied, i.e., start of 24-hour collection period. (The nitisinone dose should be taken at the normal time, irrespective of when the patient arrives at the clinic, and the time of intake recorded in the CRF.)
Collect all urine during the following 24 hours.
Record concomitant medications and adverse events since the previous visit.
Perform physical examination (as per Visit 1), 12-lead ECG and record vital signs.
Perform corneal eye assessment for signs of tyrosine toxicity.
Perform pregnancy test (premenopausal unsterilized female patients).
Perform all assessments required to generate AKUSSI (Eye and ear pigmentation, Prostate and renal stones, Osteopenia of the hip, Adult fractures, Tendon, ligament and muscle ruptures, Aortic stenosis and sclerosis, Hearing impairment, Dark eardrum, Joint and spine pain, Osteoarticular disease of joints and spine, Kyphosis and scoliosis, Arthroscopies and joint replacements). Perform other clinical assessments which are not part of the AKUSSI (Magnetic resonance imaging of spine and target joints, Knee radiographs, Ear cartilage biopsy, Range of motion of spine and joints and related rheumatology assessments, SF-36 Health Survey, Knee injury and Osteoarthritis Outcome Score [KOOS], Health Assessment Questionnaire [HAQ]). Assessments may continue on Days 2-3 as required, but must not interfere with sample collection.
Days 2-3
On Day 2, collect fasting predose serum samples for tyrosine, HGA, nitisinone and biomarkers, plasma for metabolomics and blood samples for hematology and clinical chemistry (patient remains fasting and does not take nitisinone dose until after sample collection).
After sample collection, administer of nitisinone and breakfast (in any order).
Empty bladder exactly 24 after start of urine collection period. This portion is included in the 24-hour urine.
Continue (also on Day 3) with assessments not completed on Day 1.
If urine collected upon arrival was not collected in the fasted state, a urine sample for biomarkers is collected before breakfast on Day 3.
Administered the last dose of nitisinone in the morning of Day 3.
Collect all remaining study medication from the patient and perform drug accountability.
Register new adverse events.
Patient is discharged.

Procedures may continue to a fourth day if required by logistics. This will not affect the sequence or extent of the procedures.

Patients will remain as in-patients at the hospital for the two or three nights of Visit 6.

7.5.1.8 Visit 7 (Follow-up visit): Month 49

A follow-up phone call will take place 1 month after intake of the last dose of study medication, at least 28 and no more than 35 days after Visit 6. The patient will be questioned about concomitant medication and adverse events.

7.5.1.9 Interim safety questionnaires: Months 6, 18, 30 and 42

Interim safety questionnaires will be completed by the patient to collect safety information specifically related to tyrosine toxicity. These will take place at the above time-points between the annual Visits and are described in Section 7.5.6.7.

7.5.2 Medical history

Medical history will include a systematic enquiry, past medical history, drug history, smoking and alcohol history, and allergies, as per standard clinical history-taking. In addition to the standard history, a number of questions specific for AKU will be asked.

7.5.3 Physical examination

A physical examination will be performed at each scheduled visit to the clinic. The examination will include head and neck, eyes, ENT (ears, nose and throat), heart, lung, chest, abdomen, skin, musculoskeletal, neurological, endocrine and lymphatic system, and measurement of weight and height.

New findings, or worsening of previously recorded symptoms, after Baseline must be reported as adverse events.

7.5.4 Demography

Demographic characteristics to be collected at Visit 1 will include date of birth, sex, and race.

7.5.5 Efficacy assessments

For those assessments which are operator-dependent, the same persons should conduct the test throughout the study, where possible. The person performing these assessments should be kept blinded as far as possible. Also, whenever possible, a central blinded assessor will evaluate the results (see Section 7.2, Discussion of study design). Echocardiography (7.5.5.3.6), isotope scans (7.5.5.3.10), X-rays (7.5.5.3.11, 7.5.8.4), photographs of eyes and ears (7.5.5.3.1), and ear cartilage biopsies (7.5.5.4.1) will be centrally evaluated by a completely blinded assessor. Also the exploratory assessments of MRI and knee radiographs (7.5.8.4) will be evaluated by a blinded assessor.

As described in Section 7.5.6.1.1, progression or worsening of underlying disease features which are already captured by the efficacy assessments will not be reported as Adverse Events (except

for SAEs). However, in order to collect information on possible progression of AKU symptoms, which would otherwise have been reported as AEs, for the yearly safety assessments by the DMC (see Section 7.5.6.9), the investigator or blinded assessor will indicate (Yes or No) if there has been a worsening in a disease feature since last visit. These answers will only be used for the DMC reports, and not included in the study report.

7.5.5.1 Urinary HGA (Primary efficacy parameter)

7.5.5.1.1 Sample collection and handling

Urinary excretion of HGA over a 24-hour period (u-HGA_{24}) will be assessed at all scheduled visits to the clinic.

Urine collection will start by emptying of the bladder (urine may be discarded or used for biomarkers and metabolomics). The last portion of the 24-hour sample will be collected when emptying the bladder exactly 24 hours following the start of the collection period.

Urine will be collected into 2.5-L bottles containing 30 mL of 5N H_2SO_4. During collection, avoid storage in bright light or warm conditions.

At the end of the collection period, the bottles will be weighed and the total weight recorded in the CRF. The weight of the empty bottles, before adding the H_2SO_4, is also recorded.

Further details regarding sample processing, labeling, transportation and storage will be given in a separate laboratory manual.

7.5.5.1.2 Analytical methods

The analyses will be performed by the Department of Clinical Chemistry and Metabolic Medicine at the RLUH.

The concentration of HGA in the urine will be measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) [26]. This method incorporates reversed phase chromatographic separation of polar compounds with elution in a gradient of increasing methanol. Tyrosine is measured in positive ionization mode with HGA in negative ion mode; detection is by multiple reaction monitoring (MRM) mode. Sample preparation is a dilution into a combined internal standard solution containing final concentrations of 0.4 µmol/L $^{13}C_6$-HGA and 2 µmol/L d_2-tyrosine in 0.1 % formic acid (v/v) in deionized water. Calibration is made by comparison to a standard curve of matrix-matched standards covering the pre-treated and treated concentrations for both analytes. Quality control materials are assayed at regular intervals within every batch. Samples will be analyzed in the Department of Clinical Chemistry and Metabolic Medicine at the RLUH.

RLUH will also determine creatinine and urea concentrations in the urine samples using standard analytical methods, in order to enable correction for possible incomplete 24-hour collections and varying intake of protein, respectively.

7.5.5.1.3 Calculations

The net weight of the collected urine will be calculated as part of the statistical analysis, using bottle weights before and after collection of the urine. If all bottles are of uniform weight, this weight will be used for all samples, otherwise the weight of the empty bottle recorded in the CRF should be used.

24-hour urinary HGA excretion (µmol) will be calculated by multiplying the concentration of HGA (µmol/L) in the sample with the volume collected over 24 hours (L). A density of 1.0 is assumed in this study, i.e., no conversion from grams to milliliters will be made.

7.5.5.1.4 Target u-HGA_{24}

A target level of < 300 µmol has been defined for u-HGA_{24} based on SONIA 1 results. This level represents an approximate 99 % reduction in the average patient. All subjects on 8 mg, and 2 on 4 mg, had u-HGA_{24} below this limit.

7.5.5.1.5 Rationale for assessment

The quantity of HGA excreted over 24 hours is the primary endpoint for the study. It is a reflection of the total HGA production during the 24 hour observation period and is expected to be reduced by the study medication, which prevents the production of HGA.

7.5.5.2 Serum HGA

7.5.5.2.1 Sample collection and handling

Measurements of serum HGA (s-HGA) concentrations will be performed at all scheduled visits to the clinic. One sample will be collected predose in fasting patients. The exact sampling time points will be recorded in the CRF.

Blood samples will be collected in non-gel serum tubes. Further details regarding sample processing, labeling, transportation and storage will be given in a separate laboratory manual.

7.5.5.2.2 Analytical method

The analyses will be performed by the RLUH.

The concentrations of HGA in the serum will be measured by LC-MS/MS [27]. This method incorporates reversed phase chromatographic separation of polar compounds with elution in a gradient of increasing methanol. Tyrosine and nitisinone are measured in positive ionization mode and HGA in the negative mode; detection is by multiple reaction monitoring (MRM) mode. An aliquot of the perchloric acid precipitated serum is diluted with 0.2 µmol/L $^{13}C_6$-HGA, 2 µmol/L d_2-tyrosine and 2 nmol/L $^{13}C_6$-NTBC in 0.1% formic acid (v/v). Calibration is made by comparison to a standard curve of matrix-matched standards. Quality control materials are assayed at regular intervals within every batch.

This method also includes determination of tyrosine and nitisinone. See Sections 7.5.6.2 and 7.5.7, respectively for these safety and pharmacokinetic variables.

7.5.5.2.3 Rationale for assessment

HGA in the serum may be oxidized to a melanin-like polymeric pigment and deposited in connective tissues, particularly cartilage, in a process termed ochronosis. This ochronosis is responsible for the debilitating clinical features of AKU. It is therefore a reasonable target for pharmacotherapy to attempt to reduce plasma HGA levels to normal or near normal levels, an approach which was endorsed by the Scientific Advice Working Party of the EMA.

Since it is the HGA in the body that causes the ochronosis, not the HGA excreted in urine, s-HGA would be a better primary efficacy variable than u-HGA_{24}. However, current analytical methods are not sensitive enough to quantify s-HGA in normal subjects. Efforts are now being made to improve the s-HGA assay by acidifying the sample shortly after collection. If successful (i.e., possible to quantify normal levels) s-HGA may later be considered the primary efficacy variable instead of u-HGA_{24}. If so, this will be described in a protocol amendment.

7.5.5.3 AKU Severity Score Index (AKUSSI) assessments

As mentioned above (see Section 5.1.4), the AKUSSI incorporates multiple, clinically meaningful AKU outcomes that can be described in a single score. All items included in the AKUSSI will be assessed at Baseline and yearly thereafter. Two types of AKUSSI will be used as secondary outcomes in SONIA 2. These are the Clinical Evaluation AKUSSI (cAKUSSI) (footnote 3) and a modified AKUSSI (mAKUSSI = cAKUSSI without pigment parameters). The latter was agreed with the CHMP during the Scientific Advice.

The AKUSSI scoring system is shown in Table 13 and details regarding the assessments are given in Sections 7.5.5.3.1 to 7.5.5.3.12. Each item will be given a score to be used in calculation of the AKUSSI, but all values for the underlying measurements will be reported in the CRF and will be used in the statistical analysis for the individual item.

The clinical AKU Severity Score Index (cAKUSSI) is comprised of the following assessments:

[3] In addition to the cAKUSSI, a questionnaire AKUSSI (qAKUSSI) was also used during development of the instrument [24]. The qAKUSSI will not be used in the current study.

Table 13 Clinical AKU Severity Score Index (cAKUSSI)

Feature		Score	Feature		Score
CLINICAL FEATURES NON-SPINE NON-RHEUMATOLOGIC					
Eye pigment					
R eye(Nasal)	Slight	4	**L eye (Nasal)**	Slight	4
	Marked	8		Marked	8
R eye(Temporal)	Slight	4	**L eye (Temporal)**	Slight	4
	Marked	8		Marked	8
Ear pigment					
Right ear	Slight	2	**Left ear**	Slight	2
	Marked	4		Marked	4
Stones					
Prostate Stones	Per episode	4	**Renal Stones**	Per episode	4
Musculoskeletal					
Osteopenia hip	Grade (T-scores) -1.0 to -1.7 -1.8 to -2.4 ≤ -2.5	 2 4 6			
Adult Fracture	Per fracture	8	**Ligament rupture**	Per rupture	8
Tendon rupture	Per rupture	8	**Muscle rupture**	Per rupture	8

Feature		Score	Feature		Score
Heart					
Aortic sclerosis		4	**Aortic valve stenosis**	Mild Moderate Severe	8 10 12
ENT					
Hearing impairment	Grade on audiometry (dB loss), per ear 21-35 (mild) 36-60 (moderate) >60 (severe)	 1 2 4	**Dark eardrum**	Per ear	6
NON-SPINE RHEUMATOLOGY					
Clinical joint pain (1 for each large joint area; hips, knees, ankles, feet, shoulders, elbows, wrists & hands - right and left sides = 14 joint areas)					Max 14
Non-spine osteoarticular disease (2 for each large joint area; hips, knees, ankles, feet, shoulders, elbows, wrists & hands - right and left sides = 14 joint areas)					Max 28
Arthroscopies					2 each
Joint replacements					4 each
SPINE RHEUMATOLOGY					
Clinical spinal pain (2 each for cervical, thoracic, lumbar, sacroiliac)					Max 8
Osteoarticular disease of the spine (4 each for pubic symphysis, ribs, sacroiliac, lumbar, thoracic, cervical)					Max 24
Kyphosis	(Cobb angles) 45-60 >60	 3 6	**Scoliosis**	(Cobb angles) 5-20 21-30 >30	 2 4 6

Procedures performed, and the method of scoring to determine the AKUSSI, are described below. Methodologies are described in more detail in the Study Handbook.

7.5.5.3.1 Eye and ear pigmentation

Method

Pigmentation will be assessed using clinical photographs.

Patients should be asked to have their hair slicked back to clearly display ears and skin.

Photographs should be taken by a professional photographer, using a digital camera, in a studio if possible, with standardized and optimal lighting and a standard distance from the subject. The photographs should include a standardized set that can be scored by image analysis software. The set should include each eye separately (looking to right, looking to left and looking straight), and each ear (with ruler as a guide to size if possible).

Where performed:

Clinical Photography facility in clinical trial centers.

Scoring and reporting

The photographs will be scored by a central blinded assessor. The assessor will report the degree of pigmentation (none/slight/marked) for each area and the result will be recorded in the CRF. The AKUSSI scores will be assigned as follows:

Eye	Nasal and temporal aspects score 4 each in each eye for slight and 8 for marked. This means a maximum score of 16 per eye, and 32 in total if both eyes and both sides of the eyes have marked pigmentation.
Ear	Pigmentation is scored as slight (2) or marked (4) in each ear separately. The maximum score is 8 if both ears have marked pigmentation.

In addition, digital images will be subjected to an exploratory quantitative digital image analysis.

7.5.5.3.2 Prostate and renal stones

Rationale for assessment

Abdominal features of AKU consist of renal/prostate stones, which require systematic examination often in asymptomatic individuals.

Abdominal and pelvis ultrasound will be used to assess renal and prostate stones, in addition to patients' own reports of episodes before the study and between visits.

Method

Patients will be asked to drink fluids to produce a full bladder to visualize the pelvic organs better. No other specific preparation is needed.

Standard medical ultrasound equipment for abdominal ultrasonography will be used.

In addition, if the patient experiences an episode of renal or prostate stones between visits, the patient will be asked to record details about this in a diary (see Section 7.5.6.8).

Where performed

Radiology departments in clinical trial centers.

Scoring and reporting

The investigator will answer Yes/No questions regarding new prostate and renal stones discovered at each visit in the CRF. In addition, the number of episodes with prostate and renal stones since the last visit reported by the patient will be recorded in the CRF. **Care will be taken to avoid double-counting of episodes detected on ultrasound and also reported by the patient, as well as to avoid repeated reporting of stones earlier observed.** The AKUSSI scores will be assigned as follows:

Patient-reported episodes	Each separate episode of stones, reported by the patient in the diary, scores 4 in the AKUSSI. This information is confirmed by the investigator at the visit.
Stones detected on ultrasound	Each asymptomatic new episode of stones detected on ultrasound (i.e., not also reported by the patient) scores 4 in the AKUSSI.

7.5.5.3.3 Osteopenia of the hip

Rationale for assessment

Since AKU invariably has spinal involvement with calcification of the discs, lumbar spine scores paradoxically increase rather than decrease with age. However, osteopenia of the hip occurs in AKU bone disease, where the expected decrease in bone density is seen, and can be used to assess AKU severity. DEXA currently is the easiest, most standardized method to determine bone density and osteoporosis risk.

Method

DEXA will be used to scan the hip. Standard protocols will be followed employing appropriate databases to generate bone density scores. The same hip will be used at all time points.

Standard protocols with patients recumbent will be performed. No special preparation required. In pre-menopausal women, before start of the procedure, a pregnancy test must have been performed and shown a negative result, to avoid subjecting women who may be pregnant to radiation.

Where performed

Radiology departments in clinical trial centers.

Scoring and reporting

The DEXA is performed by an independent assessor. Since the assessor will be in direct contact with the patient, there is no guarantee that the assessor is kept blinded to the treatment allocation. However, the test itself is an objective measurement, so it is unlikely that knowledge about the

patient's treatment will introduce any bias in the results. Also, the patient should be encouraged not to mention to the assessor if he/she is receiving treatment or not.

The scoring is determined using T-scores, as below.

The T-score is a comparison of a person's bone density with that of a healthy 30-year-old of the same sex. A lower T-score (more negative) means lower bone density. A T-score of -2.5 or less qualifies as osteoporosis. A T-score of -1.0 to -2.5 indicates osteopenia.

The AKUSSI scores will be assigned as follows:

Osteopenia is graded according to the T-scores:	-1.0 to -1.7 scores	2
	-1.8 to -2.4 scores	4
	≤ -2.5 scores	6

The T-score will be entered into the CRF. In addition, the bone density of the hip (g/cm^2) will be entered into the CRF.

The investigator will indicate in the CRF if there has been a clinically significant worsening since last visit.

7.5.5.3.4 Adult fractures

Method

Fractures will be reported by the patient. At the screening visit, the patient will be asked about any fractures occurring from age 18 and later. The patient's medical records, if available may also be used as a source. New fractures occurring after study start should be reported by the patient in a diary (see Section 7.5.6.8).

Scoring and reporting

At each visit, the investigator will enter information about the number of fractures since last visit in the CRF, based on the patient's diary notes. An AKUSSI score of 8 will be assigned for each fracture.

7.5.5.3.5 Tendon, ligament and muscle ruptures

Method

All ruptures will be reported by the patient. At the screening visit, the patient will be asked about any previous ruptures. The patient's medical records, if available may also be used as a source. New ruptures occurring after study start should be reported by the patient in a diary (see Section 7.5.6.8).

Scoring and reporting

At each visit, the investigator will enter information about the number of affected tendons, ligament or muscles in the CRF, based on the patient's diary notes. An AKUSSI score of 8 will be assigned for each rupture.

7.5.5.3.6 Aortic stenosis and sclerosis

Rationale for assessment

Patients with AKU have ochronosis of the heart and the great vessels especially in the aortic root. Aortic valve stenosis or sclerosis is frequently seen in this cohort. Aortic stenosis is associated with increased mortality in AKU. Left ventricular hypertrophy can occur secondary to aortic valve disease. Aortic regurgitation and mixed aortic stenosis/regurgitation can occur. Aortic and left ventricular dilatation and hypertrophy can be present.

Method

Transthoracic echocardiography will be used to assess aortic stenosis and sclerosis.

No special preparation required. Patients will need to undress to the waist and lie on the couch. Patients need to be generally well with no perturbations of blood volume. Note may need to be taken of any concomitant medication that could affect volume status, such as diuretics.

A probe is placed on the chest. Also, lubricating jelly is put on the chest so the probe makes good contact with the skin. The probe is connected by a wire to the ultrasound machine and monitor. Pulses of ultrasound are sent from the probe through the skin towards the heart. The test is painless and takes about 15-30 minutes. Patients may have to turn on their side during the test so that the operator can scan the heart from different angles. All echocardiograms will be performed according to BSE Guidelines for Valve Quantification [28].

Where performed

In the cardio-respiratory outpatient department in trial centers.

Scoring and reporting

The echocardiography is evaluated by a central blinded assessor.

In addition to the categorical stratifications of aortic valve involvement as described earlier, both the peak aortic velocity and the aortic valve area, calculated by the continuity equation method will be employed for assessment. These are reproducible measures of aortic valve involvement and are good longitudinal measures within an individual patient.

Aortic sclerosis and stenosis may be defined in terms of the visual appearance, peak velocity through the valve (AV Vpeak), valve area (AVA), mean pressure drop across the valve and the Doppler velocity index (DVI), as detailed in the table below.

Index	Normal[1]	Sclerosis	Mild[2]	Moderate[2]	Severe[2]
Visual appearance	Normal	Slightly thick/ echobright No restriction	Thickened Reduced opening		
AV Vpeak (m/s)	<2.0	<2.0	<2.9	3.0-3.9	>4.0
AVA (cm^2)	3.0-4.0		1.5-2.0	1.0-1.4	<1.0
Mean pressure drop (mmHg)			<25	25-40	>40
DVI			>0.5	0.25-.50	<0.25

1. Values taken from ACC/AHA 2006 Guidelines for the Management of Patients with Valvular Heart Disease [29]
2. Values taken from the BSE Guidelines for Valve Quantification [28].

AV Vpeak: Peak velocity through the valve AVA: Valve area DVI: Doppler velocity index

The values for the visual appearance, AV Vpeak, AVA, mean pressure drop across the valve, and the DVI will be entered in the CRF, along with the blinded evaluator's overall assessment of stenosis/sclerosis. This is mainly based on the AV Vpeak values.

The AKUSSI scores will be assigned as follows:

Normal	Scores	0
Aortic sclerosis	Scores	4
Aortic stenosis	Mild stenosis scores	8
	Moderate stenosis scores	10
	Severe stenosis scores	12

The blinded assessor will indicate if there has been a clinically significant worsening since last visit, and the answer will be recorded in the CRF.

7.5.5.3.7 Hearing impairment

Rationale for assessment

Hearing loss has been reported as a feature of alkaptonuria. The malleus, incus and the stapes are involved in synovial articulations and transmit sound waves to the inner ear. Alkaptonuria can affect these articulations and cause conduction deafness. The tympanic membrane is fibrous and can become ochronotic and stiffer. This could also affect its function in sound transmission to the inner ear. High frequency hearing loss has been noted in AKU. This will be evaluated by audiometric assessment of hearing.

Method

The audiometric test is carried out using automatic audiometers, according to standard procedures at each center. Air conduction is tested for both of the subject's ears as a minimum at the following frequencies: 0.5, 1, 2, 4, and 8 kHz, and hearing loss (dB) recorded for each frequency. If there is a reduced hearing threshold on air conduction, bone conduction is as a minimum performed at 500Hz, 1kHz, 2kHz and 4kHz.

Where performed

In the ENT services in clinical trial centers.

Scoring and reporting

The hearing impairment is assessed by an independent assessor. Since the assessor will be in direct contact with the patient, there is no guarantee that the assessor is kept blinded to the treatment allocation of the patient. However, the test itself is an objective measurement of the hearing, so it is unlikely that knowledge about the patient's treatment will introduce any bias in the results. Also, the patient should be encouraged not to mention to the assessor if he/she is receiving treatment or not.

For each ear, the grade of hearing impairment at the frequencies listed above will be entered into the CRF. The AKUSSI score will be calculated based on the average dB loss across these frequencies (air conduction). The AKUSSI scores will be assigned as follows:

Grade on audiometry (dB loss)	21-35 (mild)	Scores 1 per ear
	36-60 (moderate)	Scores 2 per ear
	>60 (severe)	Scores 4 per ear

The assessor will indicate if there has been a clinically significant worsening since last visit, and the answer will be recorded in the CRF.

7.5.5.3.8 Dark eardrum

Rationale for assessment

As mentioned above (Section 7.5.5.3.7) the malleus, incus and the stapes are involved in synovial articulations and transmit sound waves to the inner ear. AKU can affect these articulations and cause conduction deafness. The tympanic membrane is fibrous and can become ochronotic and stiffer. The eardrum will therefore be inspected for any signs of pigmentation.

Method

The inspection will be done using an otoscope. This assessment cannot be blinded, but the patient should be encouraged not to mention to the assessor if he/she is receiving treatment or not.

Scoring and reporting

The assessor will answer a Yes/No question about dark eardrum in the CRF. An AKUSSI score of 6 per affected ear will be assigned to any Yes responses.

7.5.5.3.9 Joint and spine pain

Method

At each visit, patients will be asked if they have pain in each of 14 joint areas; hips, knees, ankles, feet, shoulders, elbows, wrist & hands (right and left side) as well as cervical, thoracic, lumbar, and sacroiliac spinal regions. The patient will be asked "During the last week, have you experienced any pain in joint X"?

Scoring and reporting

The investigator will record the patient's replies to Yes/No questions for each of the 14 joint areas as well as the 4 spinal regions in the CRF. The AKUSSI scores will be assigned as follows:

Non-spine joint pain	Score of 1 for each large joint with pain. Maximum score = 14.
Spine joint pain	Score of 2 for each of regions with pain (cervical, thoracic, lumbar, and sacroiliac spinal regions). Maximum score = 8.

The investigator will indicate in the CRF if there has been a clinically significant worsening since last visit.

7.5.5.3.10 Osteoarticular disease of joints and spine

Rationale for assessment

Tc^{99m} MDP scan is a useful method for assessing the areas of involvement of the spine and joints, as this technique identifies osteoblastic changes and/or inflammatory joint pathology. In order to obtain a complete picture, both spine and non-spine joint involvement need to be assessed.

Method

It is inadvisable to perform this test if patients are volume depleted. In pre-menopausal women, before start of the procedure, a pregnancy test must have been performed and shown a negative result, to avoid subjecting women who may be pregnant to radiation.

Patients will be assessed for any existing osteoarticular disease by either Tc^{99m} MDP (methylene diphosphonate) or PET/CT employing sodium fluoride (F^{18}) scintigraphic osteoarticular scanning.

Tc^{99m} MDP: 600 MBq of methylene diphosphonate labeled with Technetium (Tc^{99m} MDP) is administered intravenously. A positive, i.e., abnormal, scan manifests as an area of increased tracer uptake and reflects the osteoblastic activity at the pathological site. The scan also provides an index of vascularity of the subchondral bone. Images are obtained 3 hours post-intravenous administration following Tc^{99m} MDP scan and 1 hour post-intravenous administration for CT and PET.

PET/CT: If more appropriate for the investigational site, these assessments may be done with PET/CT scans. 200MBq Sodium Fluoride (F^{18}) is administered intravenously.

Where performed

Nuclear Medicine facilities in clinical trial centers.

Scoring and reporting

The results are evaluated by a central blinded assessor. The assessor will answer a Yes/No question regarding the involvement of each joint and spinal region and the result will be entered in the CRF. The AKUSSI scores will be assigned as follows:

Non-spine joint involvement	Score of 2 for each large joint involvement*. Maximum score = 28.
Spine joint involvement	Score of 4 for each of pubic symphysis, ribs (costal cartilages including sternum), sacro-iliac, lumbar, thoracic and cervical regions. Maximum score = 24.

* The same 14 joints as listed in Section 7.5.5.3.9

The scoring for the assessments in the AKUSSI is the same for Tc^{99}m MDP and PET/CT scan.

The blinded assessor will indicate if there has been a clinically significant worsening since last visit, and the answer will be recorded in the CRF.

All digital images will be subjected to exploratory quantitative digital image analysis.

7.5.5.3.11 Kyphosis and scoliosis

Rationale for assessment

AKU invariably has spinal involvement, which may lead to kyphosis and scoliosis. This will be assessed using standing X-rays that includes the whole spine and pelvis – the so called stitched spine. Standing increases angles due to weight bearing.

Method

Standard protocols with patients standing will be performed. No special preparation required. In pre-menopausal women, before start of the procedure, a pregnancy test must have been

performed and shown a negative result, to avoid performing an X-ray in women who may be pregnant.

AP and lateral, stitched spine with pelvis will be obtained.

Where performed

Radiology departments in the clinical trial centers.

Scoring and reporting

The X-ray images will be evaluated by a central blinded assessor. Kyphosis will be measured from T4 to T12. Scoliosis will be measured at the worst angle, i.e., between the two end vertebrae that tilt most severely towards the concavity of the curve. For both kyphosis and scoliosis, the Cobb angles will be reported in the CRF. For patients with scoliosis (Cobb angle > 5) the two end vertebrae (upper and lower limits of the curve) will also be recorded in the CRF. The AKUSSI scores will be assigned as follows:

Kyphosis	(Cobb angles)		Scoliosis	(Cobb angles)	
	45-60	3		5-20	2
	>60	6		21-30	4
				>30	6

The assessor will indicate if there has been a clinically significant worsening since last visit, and the answer will be recorded in the CRF.

All digital images will be subjected to exploratory quantitative digital image analysis.

7.5.5.3.12 Arthroscopies and joint replacements

At the screening visit, the patient will be asked about previous arthroscopies and joint replacements. Any new episodes during the study should be recorded by the patient in a diary.

Scoring and reporting

At each visit, the investigator will enter information about location of any arthroscopies and joint replacements in the CRF, based on the patient's diary notes and an interview with the patient at the visit (see Section 7.5.6.8). An AKUSSI score of 2 will be assigned for each arthroscopy and 4 for each joint replacement.

7.5.5.4 Other clinical assessments and questionnaires

Procedures performed are described below. Methodologies are described in more detail in the Study Handbook.

7.5.5.4.1 Ear cartilage biopsy

An ear cartilage biopsy will be taken at Baseline and at the 4-year visit.

Rationale for assessment

Pigmentation of the ear cartilages is one of the earliest detectable signs of tissue ochronosis and its evolution appears to mirror the severity of joint disease in AKU. Accurate quantitation of the extent of cartilage ochronosis can be undertaken in biopsies of ear cartilage. A comparison of any pigmentation in the cartilage pre and post starting the nitisinone medication may provide important information on the effect of nitisinone on the accumulation of ochronotic pigment.

Method

The procedure will be explained and an information sheet provided to the patient at least a day before the procedure. Further explanation can be provided as required. A formal consent will be taken for the procedure.

Local anesthetic (lignocaine) is injected into the back of the ear and a small 4-mm diameter biopsy of cartilage is then taken from the conchal bowl of the ear using a posterior approach. The tissue sample is fixed in 10% PBFS (phosphate buffered (pH 7.0) 10% formal saline). A single stitch will be used and a small simple wound dressing applied. It is important that the area is kept clean and dry for at least a few days. The stitch should be removed after 7 days and this can be done by the doctor or nurse in the locality where the patient lives. The local anesthetic may cause a stinging sensation but will wear off. Most patients find this procedure is not painful however, some of the younger patients may complain of the area being painful, once the local anesthetic has worn off. Pain control for this can be discussed with the doctors. Patients who take a daily dose of aspirin or warfarin may find the wound bleeds more and for longer than those who are not taking this medication. The local anesthetic used contains adrenaline which will help minimize bleeding.

A preliminary assessment of ochronosis is made by examining biopsies on an Olympus SZH dissecting microscope using dark field illumination. Signal intensity, which is inversely correlated with pigmentation, is measured against reference standards. Digital images are captured and analyzed using Image J. Subsequently the ear cartilage is processed for histology. Serial 5-µm sections paraffin sections are prepared and stained with H&E and Schmorl's stain. It has previously been shown that Schmorl's stain is a sensitive method for detection of ochronosis (23). Sections will be analyzed on a Nikon ECLIPSE Ci-E microscope and the extent of ochronosis assessed using NIS-Elements BR Software. Ochronosis will be measured on a continuous scale, expressed as a percentage of tissue sample that is ochronotic (0-100%).

Where performed

Biopsies are taken, and samples fixed, in the trial centers and analyzed at the Clinical Research Facility, RLUH. Procedures for storing and shipping the biopsies to the RLUH will be described in the laboratory manual.

Reporting

The analysis will be performed by a central blinded assessor. The percentage of tissue sample that is ochronotic will be entered into the CRF.

7.5.5.4.2 Range of motion of spine and joints and related rheumatology assessments

Range of motion will be assessed at Baseline and yearly thereafter.

Rationale for assessment

Disability and loss of physical activity is a major factor in AKU. The effect of any treatment such as nitisinone on human movement is clinically important to assess and quantitate.

Method

Range of motion: will be measured, on right and left side, during active and passive movements and include flexion, abduction and lateral rotation in hip and shoulder, flexion and extension at the knee wrist joints and flexion, extension and abduction (radial and ulnar deviations) at the ankle. The measurements will be performed using a goniometer following appropriate positioning of the patient.

Based on the measured ranges (degrees) for the respective joint, the mean percentage loss of normal range will be calculated. Normal ranges (degrees) for the measured joints are as follows:

Joint	**Flexion**	**Abduction**	**Lateral rotation**	**Extension**	**Inversion**	**Eversion**
Hip	0 - 125	0 - 45	0 - 45	NA	NA	NA
Shoulder	0 - 180	0 - 90	0 - 90	NA	NA	NA
Knee	0 - 130	NA	NA	120 - 0	NA	NA
Ankle	0 - 20	NA	NA	0 -50[1]	0 -35	0 -25
Wrist	0 - 90	0 – 25/ 0 - 65 [2]	NA	0 - 70	NA	NA

NA: Not applicable
[1] Plantar flexion
[2] Radial deviation / Ulnar deviation
Source: http://sportsmedicine.about.com/od/glossary/g/Normal-ROM.htm

Modified Schober test for lumbar spine assessment: With patient standing in upright posture (feet hip width apart) a line 15 cm long is marked off above the midpoint of the posterior superior iliac spines. Then patient flexes forward as much as possible and the marked off line is remeasured. More than 7 cm change is normal.

For global assessment of motion, two assessments will be made.

1. 20-meter walk test: Measure the time taken to walk a fixed distance of 20 meters. The procedure to be followed is adapted from the UK NHS Operations Manual and will be included in the Study Handbook.
2. 6-minute walk test: Distance covered in a fixed time (6 minutes) will be measured. The procedure to be followed is adapted from the American Thoracic Society Guidelines and will be included in the Study Handbook.

Where performed

By a rheumatologist or physiotherapist in clinical trial centers. The assessments will be performed at each center under standardized conditions.

Reporting

- Range of motion is expressed as degrees on goniometry.
- The Schober test is expressed in centimeters.
- The 20-meter walk test is expressed in seconds.
- The 6-minute walk test is expressed in meters.

The results will be entered by the evaluator directly in the CRF. For the 20-minute walk test, the Viedoc system calculates the average speed. For the 6-minute walk test, the distance covered is calculated in percent of the predicted distance covered [30]. This is calculated as follows:

Males: Predicted distance = $(7.57 \times \text{height}_{cm}) - (5.02 \times \text{age}) - (1.76 \times \text{weight}_{kg}) - 309$ m.

Females: Predicted distance = $(2.11 \times \text{height}_{cm}) - (2.29 \times \text{weight}_{kg}) - (5.78 \times \text{age}) + 667$ m.

The investigator will indicate in the CRF if there has been a clinically significant worsening since last visit.

7.5.5.4.3 SF-36 Health Survey

The SF-36 questionnaire will be completed at Baseline and yearly thereafter.

Rationale for assessment

AKU causes significant physical disability that requires a systematic approach to assessment.

The SF-36 is a multi-purpose, short-form health survey with 36 questions [31]. It yields an 8-part profile of functional health and well-being scores as well as psychometrically-based physical and mental health summary measures and a preference-based health utility index. The eight sections are vitality, physical functioning, bodily pain, general health perceptions, physical role functioning, emotional role functioning, social role functioning and mental health.

The SF-36 is a generic measure, as opposed to one that targets a specific age, disease, or treatment group.

Method

This is a questionnaire survey to determine patient's health. This will be made available in the patient's own language. No other special preparation is required.

The SF-36 Health Survey Version 2.0 (1996) will be used. The SF-36 is suitable for self-administration, computerized administration, or administration by a trained interviewer in person or by telephone, to persons age 14 and older. In this study, the questionnaire will be self-administered.

Where performed

Self-administered by the patient while on site.

Reporting

Scoring will be performed in accordance with the SF-36 instructions.

7.5.5.4.4 Knee injury and Osteoarthritis Outcome Score (KOOS)

The Knee injury and Osteoarthritis Outcome Score (KOOS) questionnaire will be completed at Baseline and yearly thereafter.

Rationale for assessment

AKU causes significant physical disability that requires a systematic approach to assessment. The KOOS questionnaire is an instrument for assessing the patients' opinion about their knee and associated problems. KOOS is widely used for research purposes in clinical trials and also extensively used for clinical purposes. KOOS is used to monitor groups and individuals over time. [32]

KOOS is intended to be used for knee injury that can result in posttraumatic osteoarthritis (OA). It is also used in knee OA. An advantage of the KOOS is the inclusion of two different subscales of physical function relating to daily life, and sport and recreation. This enhances the instrument's validity for patients with a wide range of current and expected physical activity levels.

KOOS is intended to be used over short- and long-term time intervals; to assess changes from week to week induced by treatment (medication, operation, physical therapy) or over years following a primary injury or OA. It is thus suitable for use in this study, where patients will be followed over 4 years.

Method

The questionnaire will be made available in the patient's own language. No special preparation is required.

The questionnaire will be self-administered and takes about 10 minutes to complete.

A KOOS consists of 5 subscales; Pain, other Symptoms**,** Function in daily living (ADL), Function in sport and recreation (Sport/Rec) and knee-related Quality of life (QOL). The previous week is the time period considered when answering the questions.

Where performed

Self-administered by the patient while on site.

Reporting

Scoring will be performed in accordance with the KOOS instructions.

7.5.5.4.5 Health Assessment Questionnaire (HAQ)

The Health Assessment Questionnaire will be completed at Baseline and yearly thereafter.

Rationale for assessment

AKU causes significant physical disability that requires a systematic approach to assessment.

The HAQ is widely used throughout the world and has become a mandated outcome measure for clinical trials in arthritis [33].

The HAQ should be considered a generic rather than disease-specific instrument. Its focus is on self-reported patient-oriented outcome measures.

Method and procedure for patients

The questionnaire will be made available in the patient's own language. No special preparation is required.

The HAQ is usually self-administered, but can also be given face-to-face in a clinical setting. In this study, it will be self-administered. The questionnaire is typically handed to patients and they are asked to complete it without additional instructions. Follow-up is sometimes needed to obtain missing data or to clarify ambiguous responses in the high-quality research data applications. The HAQ Disability Index and Pain Scale can be completed in approximately five minutes.

The Short HAQ (rather than the Full Five-Dimension HAQ) will be used, which includes only the HAQ disability index (HAQ-DI) and the HAQ's patient and global pain VAS.

Where performed

Self-administered by the patient while on site.

Reporting

Scoring will be performed in accordance with the HAQ instructions.

7.5.6 Safety assessments

There is considerable clinical experience with nitisinone in the treatment of HT-1, but clinical experience in AKU is limited. The benefit/risk assessment of nitisinone for patients with HT-1,

which is a multisystem life-threatening disease which untreated leads to death at a very young age, may be different from AKU, which is slowly progressive and not life-threatening. Therefore, this long-term study will closely monitor safety, including specific assessments for the more serious adverse effects such as ocular toxicity.

All patients treated with nitisinone are expected to have serum tyrosine levels well above normal. However, most patients with elevated tyrosine do not develop clinical manifestations of toxicity. The best guide to potential toxicity is therefore close monitoring of the patients for relevant signs and symptoms, whereupon the patient will be withdrawn from the study (see Section 7.3.3).

It should be noted that the doses used in the treatment of HT-1 (1 to 2 mg/kg/day) are considerably higher than in the current study.

7.5.6.1 Adverse events

7.5.6.1.1 Definitions

Adverse event

An Adverse event (AE) is any untoward medical occurrence in a patient or trial subject administered a pharmaceutical product; the event does not necessarily have a causal relationship with the treatment or usage.

Adverse events include the following:

- Abnormal test findings, as specified below.
- Clinically significant signs and symptoms.

In this study, progression/worsening of underlying disease features which are already captured by the efficacy assessments (see Section 7.5.5) will not be reported as Adverse Events, unless it meets the criteria for a Serious Adverse Event (as defined later in this Section). Other signs and symptoms of disease progression which are not captured in the AKUSSI will be reported as Adverse Events.

In addition, signs and symptoms resulting from the following should also be handled according to the same principles as Adverse Events:

- Overdose.
- Withdrawal of treatment.
- Interactions.
- Abuse.
- Misuse.

Abnormal test findings

An abnormal test finding, e.g., abnormal laboratory analysis results, vital signs, physical examination, echocardiography (other than aortic sclerosis and stenosis) or ECG, should be recorded as an Adverse Event in any of the following situations:

- The test is associated with accompanying symptoms. Note, that the symptom, not the test result, should be recorded as an AE.
- The test result leads to a medical/surgical intervention including withdrawal of IMP or discontinuation from the study. Repeat/confirmatory testing is not considered a medical intervention.
- The investigator considers the test result to be clinically significant.

Pre-existing conditions

A pre-existing condition (i.e., a disorder present before the adverse event reporting period started and noted on the pre-treatment medical history or physical examination form) should not be reported as an adverse event unless the condition worsens or episodes increase in frequency during the adverse event reporting period.

Procedures

Diagnostic and therapeutic non-invasive and invasive procedures, such as surgery, should not be reported as adverse events. However, the medical condition for which the procedure was performed should be reported if it meets the definition of an adverse event. For example, an acute appendicitis that begins during the adverse event reporting period should be reported as the adverse event and the resulting appendectomy entered in the comments section of the CRF.

Serious adverse event (SAE)

An adverse event that meets one or more of the following criteria/outcomes is classified as serious:

- Results in death.
- Is life-threatening (i.e., at immediate risk of death).
- Requires in-patient hospitalization or prolongation of existing hospitalization.
- Results in persistent or significant disability/incapacity.
- Is a congenital anomaly/birth defect (i.e., in an offspring to the study subject).

Other medically important adverse events that may not result in death, be life threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject or may require medical or surgical intervention to prevent one of the outcomes listed above. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm, blood dyscrasias or convulsions that do not result in hospitalization or development of dependency or abuse.

Serious also includes any other event that the investigator or company judges to be serious. Any suspected transmission of an infectious agent via IMP shall also be considered serious.

Hospitalization

Hospitalization includes transfers within a hospital (e.g., from the psychiatric unit to the intensive care unit) and also includes admissions less than 24 hours. The following situations are not considered hospitalizations (although other SAE criteria may still apply):

- Outpatient procedures / ambulatory care.
- Emergency department visits.

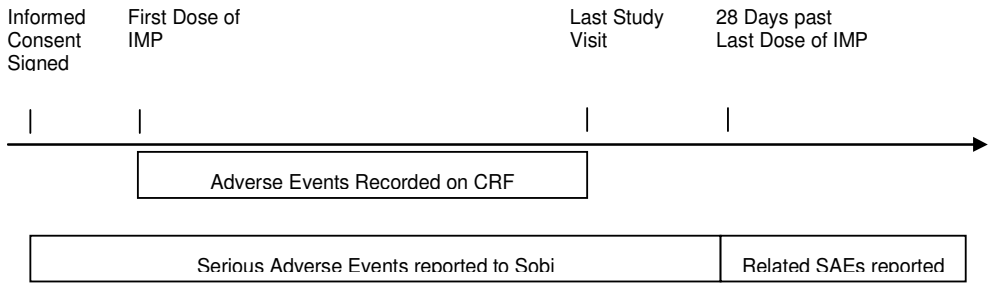
Hospitalization in the absence of an adverse event occurring during the study should not be considered an SAE. This includes:

- Hospitalization due to a pre-existing condition not associated with a worsening of the pre-existing condition.
- Protocol specified admission.
- Elective admission, e.g., due to cosmetic surgery.
- Pre-planned admission for a condition specified at Baseline for the patient.

7.5.6.1.2 Adverse event reporting period

The period for recording adverse events, including SAEs, on the CRF begins upon receiving the first dose of investigational medication, or the corresponding time for untreated patients (Visit 1) and ends at the last study visit (Visit 7).

In addition, SAEs should be reported to Sobi from the time the subject has signed the informed consent until 28 days past the last dose of IMP, or the corresponding time for untreated patients. Furthermore, any SAE should be reported to Sobi irrespective of the time of occurrence if a causal relationship between the event and the IMP is suspected.



7.5.6.1.3 Eliciting and recording adverse event information

The investigator is to record all directly observed adverse events, and all adverse events spontaneously reported by the patient, in the CRF using concise medical terminology. In addition, each patient will be questioned about adverse events at each clinic visit following initiation of treatment. The question asked will be "Have you had any health problems since your last clinic visit?"

When possible and appropriate, a diagnosis rather than individual signs and symptoms shall be recorded. The investigator is responsible for obtaining sufficient information to determine seriousness, causality and outcome of each adverse event.

Severity assessment

The investigator will use the adjectives MILD, MODERATE, or SEVERE to describe the maximum intensity of the adverse event. For the purpose of consistency, these intensity grades are defined as follows:

MILD	Does not interfere with patient's usual function
MODERATE	Interferes to some extent with patient's usual function
SEVERE	Interferes significantly with patient's usual function

Note the distinction between the gravity (seriousness) and the intensity (severity) of an adverse event. **Severe** is a measure of intensity; thus, a **severe** reaction is not necessarily a **serious** reaction. For example, a headache may be severe in intensity, but would not be classified as serious unless it met one of the criteria for serious events listed above.

Causality assessment

For each adverse event, the investigator must make a causality assessment to determine if there is a reasonable possibility that the IMP caused the adverse event. The adverse event is assessed as related or not related to the IMP. Causality should be graded as related/not related.

7.5.6.1.4 Serious adverse event reporting

Both serious and non-serious adverse events are to be reported on the adverse event page of the CRF as specified in the CRF instructions.

If an SAE occurs, Drug Safety at Sobi is to be notified by e-mail to drugsafety@sobi.com or by fax to +4686973230, using the designated Serious Adverse Event Form within 24 hours of awareness of the event by the investigator.

Where the same data are collected in the CRF and on the SAE form, these must be completed in a consistent manner. For example, the same adverse event term should be used on both forms.

All new information obtained, relevant to an SAE report, should be forwarded to Sobi within the same timeframe as the initial information.

The investigator shall provide Sobi with sufficient information to enable a complete medical assessment of the reported event. Best efforts shall be made by the investigator to provide Sobi with additional information related to any SAE as requested.

7.5.6.1.5 Exposure during pregnancy

All events of exposure to the IMP during pregnancy (female patient or male patient's partner) shall be reported to Sobi (fax number + 46 8 697 32 30 or by e-mail to drugsafety@sobi.com) within 24 hours of awareness by any study personnel, whether the exposure is associated with an adverse event or not. This includes all situations where a female is or has been found to be pregnant after being exposed to IMP; directly, indirectly or via her partner (paternal exposure).

In all reported situations of exposure during pregnancy, Sobi will provide the investigator with a Pregnancy Report Form which shall be completed and returned by the investigator. The investigator is responsible for monitoring the outcome of the pregnancy and to inform Sobi of relevant information and any information requested related to the outcome of the pregnancy.

Any adverse events and SAEs observed during and in relation to pregnancy or delivery should be recorded in the CRF and as applicable be reported to Sobi as described previously in this section.

7.5.6.1.6 Rescue procedures in case of tyrosine-related adverse events

Patients will be provided with information regarding possible signs and symptoms which can occur as a result of elevated tyrosine, secondary to nitisinone treatment. Elevated tyrosine may lead to ocular signs and symptoms, including corneal ulcers, corneal opacities, keratitis, conjunctivitis, eye pain, and photophobia. It may also lead to hyperkeratotic skin lesions.

All patients will be provided with information at the start of the study with instructions about what to do in the event that they develop a skin rash or photophobia, eye pain, or signs of inflammation such as redness, swelling, or burning of the eyes during the study. These instructions will be to:

- Immediately contact the investigator (24-hour telephone number given in the Patient Information Sheet) and visit the investigational site as soon as possible for corneal eye examination, measurement of serum tyrosine concentration and safety hematology and clinical chemistry.

In case a patient develops signs or symptoms of tyrosine toxicity, this will be reported as an adverse event and followed up as detailed below (Section 7.5.6.1.7; Follow-up of adverse events).

Nitisinone should be withdrawn in patients who develop signs of tyrosine toxicity. If feasible, once the symptoms have resolved, nitisinone may be reintroduced at a lower dose (2 mg). Alternatively, the patient is withdrawn from the study. If ocular tyrosine-related symptoms reappear on the lower dose, nitisinone should be withdrawn and the patient monitored until the symptoms resolve. Nitisinone should then not be reintroduced again.

Patients who are temporarily withdrawn due to a suspected, but not later confirmed, tyrosine toxicity, may continue in the study on the 10-mg dose.

7.5.6.1.7 Follow-up of adverse events

All adverse events should be followed until they are resolved or the investigator assesses them as chronic or stable, or the patient's participation in the study ends, i.e., until the follow-up visit. How to report changes in an on-going adverse event during a patient's participation in the study is described in the CRF instructions.

In addition, all serious and non-serious adverse events assessed by the investigator as related to the IMP will continue to be followed until they resolve or until the investigator assesses them as "chronic" or "stable", even after the patient's participation in the study is over.

7.5.6.2 Tyrosine

Serum tyrosine will be determined at all scheduled visits to the clinic. It is determined in the same sample, and using the same analytical method as s-HGA. See Section 7.5.5.2 for details.

7.5.6.3 Laboratory safety assessments

The full range of laboratory tests (clinical chemistry and hematology) will be performed at all scheduled visits to the clinic. Additional measurements will be made in case of adverse events, if relevant.

The following will be measured:

Hematology (blood):

Hemoglobin, hematocrit, leukocytes and differential, platelets, mean corpuscular volume (MCV) and erythrocyte sedimentation rate (ESR).

Clinical chemistry (serum):

Total bilirubin, alkaline phosphatase, gamma-glutamyltransferase (gamma-GT), alanine aminotransferase (ALT/SGPT), creatinine, urea, total protein, albumin, glucose, inorganic phosphate, sodium, potassium, calcium, and chloride.

In case of any hepatic values outside of the reference range, all hepatic tests should be repeated and aspartate transaminase (AST/SGOT) should be included.

Clinically significant abnormalities will be recorded. In case of relevant changes after treatment initiation, these will be reported as adverse events (see Section 7.5.6.1.1 for details).

7.5.6.4 Electrocardiogram (ECG)

Procedure for patients: No special preparation required. All patients will have a resting ECG.

Method: A resting standard 12-lead electrocardiogram will be recorded at all scheduled visits to the clinic.

Where performed: This will be performed in the cardio-respiratory outpatient department, or other appropriate site, in clinical trial centers.

Calculations if any: Routine characteristics of the ECG will be calculated as part of the automated analysis of the instrument. These will include rate, rhythm, and components of the ECG waveforms that include durations and other abnormalities.

7.5.6.5 Vital signs

Vital signs (blood pressure, pulse rate, temperature) will be recorded at all scheduled visits to the clinic. Additional measurements will only be made in case of adverse events, if relevant.

Systolic and diastolic blood pressure and pulse rate will be recorded after 5 minutes of rest in the supine position. The results will be recorded in the CRF.

Temperature will be measured using the standard method at the investigative site.

Clinically significant abnormalities will be recorded. In case of relevant changes after treatment initiation, these will be reported as adverse events (see Section 7.5.6.1.1 for details).

7.5.6.6 Corneal ocular examination

A corneal eye examination will be performed at all scheduled visits to the clinic. This will consist of corneal photographs with or without slit-lamp examination, as well as visual acuity testing if relevant. In case of relevant changes after treatment initiation, these will be reported as adverse events (see Section 7.5.6.1.1 for details).

Procedure for patients: No special preparation required.

Method: The procedure uses an anterior segment camera. Low and high magnification images are made of any corneal opacities.

Where performed: At ophthalmological services in clinical trial centers.

Rationale for assessment: Nitisinone therapy increases circulating tyrosine concentrations. For the purposes of patient safety, it is necessary to check the cornea at every visit to ensure there is no keratopathy, even if there are no symptoms.

7.5.6.7 Safety monitoring questionnaire

Patients will complete a safety monitoring questionnaire between the scheduled site visits, at months 6, 18, 30 and 42 in order to collect safety information specifically related to tyrosine toxicity. The patients will be asked if they have had any problems with their eyes or skin since their last visit to the study site. In such a case, they will be asked to describe the problem and when it occurred.

Telephone contact with the patient will be made if indicated by the response to these questions.

7.5.6.8 Patient diary

The information from the patient diaries will be used to assist the investigator in their AKUSSI assessment and will not be separately analyzed.

Patients will be asked to record information about any emerging AKUSSI-related events. Patients will be asked to record information (event and date, treating physician's name and institution) on the following items as they occur:

- Fractures.
- Muscle/tendon/ligament ruptures.
- Renal stones.
- Prostate stones.
- Arthroscopies.
- Joint replacements.

7.5.6.9 Data monitoring committee

An independent data monitoring committee (DMC) will continuously monitor the safety of the study by receiving copies of all SAE reports. The DMC will once yearly be provided with all safety data, including events captured as part of the efficacy assessments and exempt from AE reporting (see Section 7.5.6.1.1), and will produce a safety report on an annual basis. The DMC will also review the interim analysis report. Further details will be described in a separate Charter.

7.5.7 Pharmacokinetic assessments

There will be no determination of pharmacokinetic variables in this study. But predose concentrations of nitisinone will be determined in the same samples, and using the same analytical method as for s-HGA. See Section 7.5.5.2 for details.

7.5.8 Exploratory efficacy assessments

7.5.8.1 Inflammatory and oxidative marker analysis

7.5.8.1.1 Sample collection and handling

Immunoassays for pro-inflammatory cytokines, chemokines, and acute phase proteins will be determined in fasted predose serum samples collected at all visits. See Sections 7.5.5.1.1 and 7.5.5.2.1 regarding sample collection and handling.

7.5.8.1.2 Measurements

The following inflammatory markers will be measured:

- Cytokine immunoassays (IL-6 and IL-8).
- Serum amyloid A.

A number of other markers may also be measured if deemed relevant. In particular, a limited subset of samples (#patient, sampling time) will be selected on the basis of the previously obtained results in order to investigate: levels of C-reactive protein (CRP), indices of oxidative

stress such as lipid peroxides and thiols, protein oxidation (protein carbonyls or thiol-oxidized proteins).

7.5.8.1.3 Analytical method

The analyses will be performed by the Department of Biotechnology, Chemistry and Pharmacy at the University of Siena.

Pro-inflammatory cytokine (IL-6)/chemokine (IL-8) serum amyloid A and CRP (in selected samples) will be analyzed using a bead-based multiplex assay (BioPlex or Milliplex).

Dedicated spectrophotometric assays will be used in selected samples to evaluate lipid peroxides and thiol levels, according to protocols already described and validated.

Protein oxidation will be investigated in selected samples by means of two-dimensional electrophoresis and Western Blot according to protocols already described and validated.

Systems using xMAP Technology (Luminex) perform discrete bioassays on the surface of color-coded beads known as microspheres, which are then read in a compact analyzer. Using multiple lasers or LED and high-speed digital-signal processors, the analyzer reads multiplex assay results by reporting multiple colors on each individual microsphere particle.

First, Luminex uses proprietary techniques to internally color-code microspheres with various fluorescent dyes. Distinctly colored bead sets can be created, each of which can be coupled with a reagent specific to a particular bioassay. After an analyte from a test sample is captured by the bead, a reporter molecule, labeled with a different fluorescent dye, is introduced to complete the reaction on the surface of each microsphere. Next, the microspheres' internal dyes are excited by the laser or LED, marking the microsphere set. A second laser or LED excites the fluorescent dye on the reporter molecule. Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay, based on fluorescent reporter signals [34]. Calibration curves from protein standards are prepared in parallel [35].

As for sample stability, degradation of cytokines is observed for IL-8 already within one year, whereas IL-6, is degraded up to 50% or less of Baseline values within 2-3 years if samples are properly stored at -80°C and do not undergo freeze-thaw cycles. If samples are stored at -20°C, degradation occurs faster [35].

7.5.8.1.4 Rationale for assessment

Inflammation and oxidation are significant components of arthritis in AKU. The assay of inflammatory and oxidative markers pre- and post-nitisinone will allow validation of these markers for future studies and for clinical practice.

7.5.8.2 Connective tissue damage marker analysis

7.5.8.2.1 Sample collection and handling

Markers for connective tissue damage (by measurement of established bone, cartilage and cardiovascular markers) will be determined in fasted predose serum and urine samples collected at all Visits. See Sections 7.5.5.1.1 and 7.5.5.2.1 regarding sample collection and handling.

7.5.8.2.2 Measurements

The following measurements are planned:

- Cartilage markers (CTX-II, C2M, aggrecan, C6M).
- Bone markers (CTX-I, PINP, N-MID osteocalcin).
- Cardiovascular and inflammatory (VCANM, TIM, MIM, CRPM).
- A number of other relevant markers may also be measured, including but not limited to markers of fibrosis (C1M, C3M).

7.5.8.2.3 Analytical method

The analyses will be performed by Nordic Bioscience.

The assays for CTX-I (β-CrossLaps/serum) and osteocalcin (N-MID osteocalcin) are sandwich ELISAs for the detection of the markers in serum, lithium heparin plasma or EDTA plasma. They are based on the principle of ECLIA (electrochemiluminescence immunoassay) and are measured on Elecsys 2010. The sandwich complex is formed by the addiction of the biotinylated monoclonal anti β-CrossLaps/N-MID Osteocalcin, with the samples and the monoclonal CrossLaps/N-MID Osteocalcin labeled with ruthenium complex. The sandwich complex is bound to the solid phase via biotin-streptavidin interaction. The reaction mixture is aspirated into the measuring cell where microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed by Procell. Application of voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier.

The assay for CTX-II (U-CTX-II) is a competitive ELISA for the detection of the marker in urine. The assays for C2M, C6M, aggrecan (AGNx-II), VCANM, TIM, MIM and CRP-M are competitive ELISAs for the detection of the markers in serum, lithium heparin plasma or EDTA plasma. The procedure for these assays follows the one described by Bay-Jensen et al. [36].

The samples are stable at -20°C for 3 months, and for longer periods when stored at -80°C.

All the validated markers will be measured in the Nordic Bioscience quality controlled laboratory in Herlev

7.5.8.2.4 Rationale for assessment

Unbalanced turnover of matrix proteins is a crucial process in arthritis, which is one of the major effects of AKU. Hence monitoring turnover of cartilage, bone and other connective tissues may

be helpful for diagnosis or efficacy-evaluation of AKU therapy. The assay of connective tissue markers pre- and post-nitisinone will allow validation of these markers for future studies and for clinical practice.

7.5.8.3 Metabolomics

7.5.8.3.1 Sample collection and handling

Metabolomics will be determined in urine and plasma collected at all Visits as follows:

Urine: An aliquot of the urine collected for HGA as described in Section 7.5.5.1.1 will be used.

Plasma: A sample will be collected a 2-mL K-EDTA tube before breakfast.

In addition, metabolomics may be determined in all available urine and serum/plasma samples.

7.5.8.3.2 Analytical method

The analyses will be performed by the RLUH.

Tandem mass spectrometry (Tandem MS) with time of flight (TOF) capability will characterize the tyrosine metabolites in serum and urine. The principal method employed to generate the pilot data uses gradient elution reversed phase HPLC coupled to highly sensitive time-of-flight mass spectrometry. As used in the pilot studies, biological samples will be analyzed using UHPLC/QTOF-MS (Agilent 1290 Infinity series coupled to an Agilent 6540 QTOF mass spectrometer) in conjunction with statistical data analysis in order to investigate and identify differences in the metabolomic profiles of healthy subjects and patients with AKU (nitisinone treatment and placebo groups). Serum and urine samples will be analyzed and characterized by their accurate mass (m/z) and retention time (RT) both in positive and negative ion modes.

7.5.8.3.3 Rationale for assessment

The changes in tyrosine pathways consequent upon AKU are still not adequately characterized. Nothing is known about changes in tyrosine pathways post-nitisinone in AKU patients beyond the fact that increases in tyrosine are seen. Understanding the metabolic pathways in AKU pre- and post-nitisinone is necessary to fully understand the long-term safety of nitisinone.

7.5.8.4 Magnetic resonance imaging of spine and target joints, and knee radiographs

Magnetic resonance imaging (MRI), and a complementary knee radiograph, will be performed at Baseline, and at the 12- and 48-month visits.

Rationale for assessment

MRI is a medical imaging technique used in radiology to visualize internal structures of the body in detail. MRI makes use of the property of nuclear magnetic resonance (NMR) to image nuclei of atoms inside the body. MRI provides good contrast between the different soft tissues of the body, which makes it especially useful in imaging the joints, tendons and muscles, compared with other medical imaging techniques. MRI does not use ionizing radiation.

Plain radiographs of the knees are the gold standard for assessment of osteoarthritis, and this will be included for those patients undergoing MRI assessments of the knee.

Method

MRI scans of thoracic and lumber spine as well as a knee and ankle joint will be carried out. It is anticipated that some patients will have had joints replaced or may have them replaced during the study period. Hence a knee and an ankle joint are being imaged since ankle joints rarely need replacing. Fractures and disc disease can also be identified by MRI. Thoracic and lumbar spine will be imaged in addition to two target joints.

No special preparation is required. Patients will be asked to answer standard MRI safety questionnaire immediately prior to scanning. In pre-menopausal women, before start of the procedure, a pregnancy test must have been performed and shown a negative result to avoid that scanning is performed in women who may be pregnant. Patients with implantable devices such as pacemakers, certain prosthetic heart valves and some types of neural stimulator will not be able to undergo MRI scanning.

Despite being painless, MRI scans can be unpleasant for those who are claustrophobic or otherwise uncomfortable with the imaging device surrounding them. Patients may have difficulty lying on their backs, such as AKU patients with spinal disease, for an hour or more without moving.

A 3T imaging protocol has been adapted for a Siemens magnet and used in RLUH. This protocol will be adapted for all clinical trial centers which will perform MRI. It uses a wrap-around multi-channel flex surface coil (such as cardiac coil or 8-channel torso coil).

Where performed

Radiology departments in clinical trial centers where it is feasible to do so.

Scoring and reporting

The MRI scans will be evaluated by a central blinded assessor.

Thoraco-lumbar spine

Each intervertebral disc space (12 thoracic, 5 lumbar) will be graded for degenerative changes using a modified Pfirrmann grading system which is an 8 point score on T2 weighted images. A total score for the T/L spine will be recorded.

Presence or absence of end plate marrow edema changes on STIR images will be recorded.

When present low (dark) signal changes on T1 images within the nucleus will be recorded.

When present high T1 signal crossing the disc space (reflecting osseous fusion) will be recorded.

The presence of insufficiency fractures of the vertebral bodies will be assessed on T1 images.

Knee MRI

One knee will be assessed using a modified WORMS grading system as follows:

- Cartilage signal and morphology - 12 sub regions (4 patello-femoral, 8 tibio-femoral) - score 0 - 6 depending on depth and extent of cartilage loss.
- Bone marrow lesions - 12 sub-regions - score 0 - 3.
- Sub-chondral cysts - 12 sub-regions - score 0 - 3.
- Osteophytes - 10 sites - score 0 - 7.
- Effusion - whole knee - score 0 - 3.
- Synovitis - intercondylar and para-patellar regions - score 0 - 3.
- Meniscal status - anterior horn, body and posterior horns, medial and lateral menisci - score 0 - 4.
- Ligaments - Cruciate and collateral ligaments - score 0 - 2.
- Loose bodies - whole knee – score 0 - 3.

Also assessed (additional to WORMS system) is the presence of low intrachondral signal on PD and T2 weighted images.

Knee radiographs will be scored by the Kellgren-Lawrence system based on 3 compartments – score 0 - 3.

Ankle MRI

One ankle will be assessed using Arthrosis Grading System (Adapted WORMS)

- Cartilage signal and morphology - 8 sub regions (4 tibial plafond, 4 talar dome) - score 0 - 6 depending on depth and extent of cartilage loss.
- Bone marrow lesions - 4 sub-regions - score 0 - 3.
- Sub-chondral cysts - 4 sub-regions - score 0 - 3.
- Osteophytes - 6 sites - score 0 - 7.
- Effusion - whole ankle - score 0 - 3.
- Synovitis - (2 response fields) anterior and posterior regions - score 0 - 3.
- Ligaments - (2 response fields) Deltoid and lateral ligaments - score 0 - 2.
- Loose bodies - whole ankle - 0 - 3.

Also assessed (additional to WORMS system) is the presence of intra-chondral low signal intensity on PD and T2 weighted images – yes / no.

All digital images will be subjected to an exploratory quantitative digital image analysis to identify changes from Baseline.

7.5.9 Genetic assessments

7.5.9.1 Sample collection and handling

Blood sampling to obtain DNA will be performed during Visit 1 by obtaining 2 mL of peripheral blood in EDTA. DNA will be extracted from the blood samples at the site where the sample is taken using an available standard method. The extraction will be in accordance with the local DNA extraction protocol.

Details regarding sample labeling and transportation will be given in a separate laboratory manual.

The DNA will be used for sequencing of the HGD gene, as described below.

7.5.9.2 Analytical method

Genetic assessments will be performed by the Institute of Molecular Physiology and Genetics. Specific Polymerase Chain Reaction (PCR) primers will be used for PCR amplification of all 14 exons (coding parts) with short flanking intronic sequences of the homogentisate 1,2-dioxygenase (*HGD*) gene from the patient's DNA. Obtained PCR products will be purified and sequenced using commercial sequencing kits and ABI PRISM® 3100-Avant Genetic Analyzer. All identified nucleotide substitutions/deletions/ insertions will be validated and identified disease causing mutations will be described according to HGVS (Human Genome Variation Society) guidelines & recommendations [37].

7.5.9.3 Output of the analysis

The patients' mutations will be included in the *HGD* mutation database [38]. Identifiers will be used which allow seeing which patients belong to the study (all data is anonymized) as follows:

Patients' alleles: AKU_TR_number a/b

Reference: DevelopAKUre

7.5.9.4 Rationale for assessment

This DNA will be used for *HGD* mutation/polymorphism detection and subsequent genotype-phenotype correlations studies.

Whilst taking the medical history (see Section7.5.2), the following specific questions will be asked:

- Is there any family history of AKU?
- If so, who is affected (relationship to the patient)?
- Does the patient or a relative suffer from other diseases affecting the joints?
- Are the parents of the patient consanguineous?
- Which is (are) the country (-ies) of origin of the patient's parents?

These questions allow other members within the same family with the same mutation to be identified. Similar genetic background and their clinical severity status can then be used in order to validate the contribution to the AKU severity of genetic factors and of possibly other pathology in the family.

The last two questions together with the analysis of the haplotypes associated with mutations also allow observing variability possibly caused by the different mutational events (different haplotypes) and geographical differences contributing to the AKU severity.

8 Quality control and quality assurance

This study will be conducted in compliance with this protocol, study specific procedures and SOPs, the ICH Guideline for Good Clinical Practice, and SOPs of the relevant parties, including but not limited to PSR's SOPs for trial and data management, Sobi's SOPs for safety data reporting to regulatory agencies, RLUH's SOPs regarding the analytical methods, and University of Liverpool SOPs for statistical analysis.

Monitoring visits to the study sites will be performed periodically during the study, to help ensure compliance with the protocol, study specific procedures and applicable regulatory requirements. Source documents will be reviewed for verification of agreement with data in CRFs. All patient informed consent forms will be reviewed. The investigators or institutions guarantee access to source documents by PSR, its representatives, and appropriate regulatory agencies.

The study sites may be subject to a quality assurance audit by PSR or its representatives, as well as inspection by appropriate regulatory agencies.

It is important that the investigators and relevant personnel are available during the monitoring visits and possible audits and that sufficient time is devoted to the process.

9 Statistical plan

9.1 Determination of sample size

Only a few subjects would be needed to detect a relevant efficacy when using u-HGA_{24} as primary endpoint. Therefore sample size has been based on the AKUSSI score to allow the possibility to establish a clinical effect as well.

Based on data from the previous cross-sectional study of AKU using AKUSSI [24], and follow-up data, if the measure of efficacy is that nitisinone reduces the mean increase in AKUSSI over the 4-year period to 4, and taking the standard deviation of the increase to be 8, then a sample size of 64 per group is required for a two sided t-test with power 80% for significance level 0.05. With a 10% drop-out rate, a sample size of 70 per group is required (140 patients in all).

9.2 Definition of study populations

The following populations will be defined:

- The Full Analysis Set (FAS) will be used for the analysis of efficacy variables. All randomized patients who have a valid u-HGA_{24} at Baseline will be included in the FAS.
- The Per-protocol analysis set (PP) will be a subset of FAS and will be used for the analysis of the primary endpoint. All randomized patients who have no important protocol violation or deviation that could affect primary endpoint will be included.
- The Safety Analysis Set will be used for the analysis of safety variables. All randomized patients will be included with the exception of patients randomized to nitisinone who never received any study medication.

9.3 Overall statistical and analytical plan

This section describes the statistical analysis as it is foreseen at the time of planning of the study. Any major deviations from this plan, reasons for such deviations and all alternative or additional statistical analyses that may be performed will be described in the statistical analysis plan (SAP). The SAP will give a detailed description of all statistical analyses. The first version of the SAP will be completed before any study data has been made available to the persons involved in the preparation of the SAP. The second version will be completed before locking the study database. The SAP will serve as a complement to the protocol and supersedes it in case of differences.

All statistical analyses will be performed with SAS System (SAS Institute, Cary, NC).

9.3.1 General statistical issues

Two-sided 95% confidence intervals corresponding to a two-sided 5% level of significance will be used throughout the analyses.

All relevant study data will be summarized graphically where appropriate and tabulated with descriptive statistics, including e.g. arithmetic and geometric mean, standard deviation, standard error of the mean, median, minimum and maximum for the continuous variables, and frequencies and proportions for the categorical variables. Both absolute values and changes from Baseline will be tabulated, if feasible. In addition to the descriptive statistics, statistical analyses will be conducted as described below.

An interim analysis will occur after all patients have completed the first 12 months (see Section 9.3.8). This will include a complete set of analyses of all variables up to 12 months.

9.3.2 Demographics and baseline characteristics

The demographic and baseline characteristics will be tabulated by treatment group using descriptive statistics.

9.3.3 Analysis of primary endpoint

The primary endpoint is u-HGA_{24} at Month 12. Based on previous data it is assumed that u-HGA_{24} will have a log-normal distribution. The primary analysis will be a mixed model repeated measurement (MMRM) as follows: log(u-HGA_{24}) = treatment, site, age category, visit, treatment-by-visit interaction where treatment, visit and treatment-by-visit interaction will be included as fixed factors while subject-within-site will be included as a random factor. A compound symmetry covariance matrix will be used. The restricted maximum likelihood method (REML) will be used and the degrees of freedoms will be estimated using Kenwood-Rogers method.

Model based point estimates and associated 95% confidence intervals will be calculated.

The LS means and confidence interval for within-group and between-group estimate will be exponentiated which will correspond to adjusted geometric means and ratio of adjusted geometric means respectively.

Data from beyond the 12 month visit will not be included in the primary analysis.

9.3.4 Sensitivity analyses for primary endpoint

The robustness of the primary analysis will be checked by conducting the following sensitivity analyses.

- Repeat of the primary analysis on the per-protocol analysis set.
- A sensitivity analysis using multiple imputation techniques for missing data. It is expected that the main reason for missing data will be drop out from the study. Thus a monotone missing pattern will be assumed.
- It is expected that drop-outs mainly will occur in the untreated population as they may feel less motivated to return to visits over time. However, should there be a substantial drop out among patients treated with nitisinone, a sensitivity analysis using a pattern-mixture approach will be considered where it will be assumed that drop-outs in the nitisinone group will follow a trajectory as in the untreated group after the time point for drop-out.

9.3.5 Analysis of secondary endpoints supporting primary endpoint

U-HGA_{24} at months 3, 24, 36 and 48 will be analyzed using the same MMRM model as in the primary analysis.

The occurrence of achieved target level of u-HGA_{24} at month 3, 12, 24, 36 and 48 will be analyzed using Fisher's exact test at each time point. It is expected that the proportion of patients in the untreated group who achieve the target level is 0 % or close to 0 %. Patients withdrawn from the study will be considered not having achieved target level for any time point after drop-out.

9.3.6 Analyses of endpoints supporting secondary objectives

Changes from Baseline in cAKUSSI, mAKUSSI, individual AKUSSI items, SF-36 domains, HAQ, KOOS, Range of motion, and predose s-HGA will be analyzed using the same MMRM model as in the primary analysis (see Section 9.3.3). Transformations will be applied to the data where necessary (e.g., log) and only done so when the model is badly fitting for the data on the raw scale. A transformation will only be chosen based on model fit (e.g. AIC and an analysis of residuals) and not on any p-values obtained for the treatment or any other effect.

Where possible, binary and ordinal secondary endpoints will be analyzed with repeated measurement models using generalized estimation equations (GEE). However, where it is clear that a variable value can never decrease (improve) from one visit to another (e.g., the number of fractures for a patient can only increase or remain the same at each visit), a randomization test will be used based on randomly assigning each individual's data series to one of the two arms and calculating a particular test statistic. This procedure will be carried out 999 times and then the true value of the statistic (i.e., with individuals in their true arms) ranked among these. Full details will be in given in the SAP.

9.3.7 Analysis of safety and tolerability data

9.3.7.1 Adverse events

All adverse events (AEs) during the study will be coded using the Medical Dictionary for Regulatory Activities (Version 17 or later updates). The number of patients with any adverse events and the total number of adverse events will be summarized in frequency tables by treatment group, system organ class and preferred term. Incidences will be expressed both as percentages of safety population as well as event rates per person-year. In addition, the number of adverse events will be evaluated by seriousness, and severity. The adverse events leading to premature discontinuation will also be summarized.

9.3.7.2 Safety laboratory parameters

The changes in safety laboratory parameters from Baseline to all post-baseline visits will be calculated and summarized by treatment group and visit using descriptive statistics. In addition, the proportions of patients with abnormal values will be tabulated by treatment group and visit.

Shift plots comparing each visit with baseline level will also be produced.

9.3.7.3 Vital signs

The changes in vital signs from Baseline to all post-baseline visits will be calculated and summarized by treatment group and visit using descriptive statistics.

9.3.7.4 Corneal eye examination

The outcome of corneal eye examination will be made by treatment group and visit using descriptive statistics.

9.3.8 Interim analysis

An interim analysis will be conducted when all patients have completed 12 months of treatment. This will include a complete set of efficacy and safety data up to 12 months. The analysis of the primary endpoint, u-HGA at month 12, will thus be covered by this interim analysis. A decision will be made whether the data supports an early submission of a Market Authorization Application. The study will continue up to 48 months (or until granting of a Marketing Authorization) irrespective of whether data allows for a submission or not, subject to the conditions given in Section 5.2.3.

9.3.9 Multiple comparison/multiplicity

No adjustment for multiple comparisons will be made.

9.3.10 Subgroup analyses

Subgroup analyses will be conducted by age, sex and race on key efficacy and safety parameters.

9.3.11 Handling of missing data

The efficacy data will be analyzed with MMRM which can handle missing data under the assumption of MAR (missing at random). As mentioned in section 9.3.3.1 sensitivity analyses that address impact of missing data will be conducted using multiple imputation methods. It is expected that the main reason for missing data will be due to drop out from the study. Thus a monotone missing pattern will be assumed. In addition, it is expected that drop-out mainly will occur in the untreated population as they may feel less motivated to return to visits over time. However, should there be a substantial drop-out among patients treated with nitisinone, a sensitivity analysis using a pattern-mixture approach will be considered where it will be assumed that drop-outs in the nitisinone group will follow a trajectory as in the untreated group after the time point for drop-out.

This will be done for both the primary endpoint u-HGA_{24} as well as for the clinical endpoints.

10 Data collection, handling and record keeping

10.1 Data standards

Collection of data should be performed in the Clinical Data Acquisition Standards Harmonization (CDASH) format, according to the Clinical Data Interchange Standards Consortium (CDISC). The standards should be used to the extent possible and/or required for the specific study/project. The minimum requirement of the CDISC standard is to collect all core variables specified as 'Required' in the Study Data Tabulation Model (SDTM) format.

10.2 Case report form

A CRF is required and should be completed for each included patient. In this study an electronic CRF will be used, created within the Viedoc system [39]. Viedoc is a fully validated and FDA-compliant Electronic Data Capture (EDC) system, with several additional features, such as Interactive Web Response System (IWRS), possibility for patients to complete questionnaires directly into the system, and the possibility for images and reports to be uploaded into the system to allow for central evaluation and scoring.

The completed original CRFs are the sole property of the University of Liverpool, and should not be made available in any form to third parties, except for authorized representatives of appropriate Regulatory Authorities, without written permission from the University of Liverpool.

It is the responsibility of the investigator to ensure completion and to review and approve all CRFs. CRFs must be signed by the investigator, using electronic signature. These signatures serve to attest that the information contained on these CRFs is correct. At all times, the investigator has final responsibility for the accuracy and authenticity of all data entered on the CRFs.

10.3 Source data

Patient source documents are the physician's patient records maintained at the study site. In most cases, the source documents will be the hospital's or the physician's chart. In those cases, the information collected on the CRFs must match those charts. All reports and printouts should be stored in the patient's medical record.

The data management plan will describe which source data are entered directly on the CRFs.

10.4 Database closure

After cleaning of all data up to and including the 12-month visit, a copy of the database (interim database) will be locked and used for the data analysis for the interim report (see Section 9.3.8).

The complete database will be locked after cleaning of all data at the end of the study.

Prior to each database lock, all tasks or criteria defined in the data management plan must be completed and documented. The study database, or interim database, must be locked before generation of any results. Each database lock will be approved by relevant study personnel. After the final database lock all edit accesses will be removed. The final study database can only be unlocked after written approval by the DevelopAKUre consortium.

10.5 Record retention

To enable evaluations and/or audits from Health Authorities or PSR, the investigator agrees to keep records in accordance with the essential documents defined in the ICH GCP Guidelines [1], including the identity of all participating patients (sufficient information to link records, e.g., CRFs and hospital records), all original signed informed consent forms, electronic copies of all CRFs and detailed records of IMP accountability. The records should be retained by the investigator according to local regulations.

If the investigator relocates, retires, or for any reason withdraws from the study, the study records may be transferred to an acceptable designee, such as another investigator, another institution, or to the Sponsor. The investigator must obtain the Sponsor's written permission before disposing of any records.

11 End of study

The end of the study is when the last patient has completed the last study visit (Visit 7) and the End of Study procedures have been completed.

12 Study discontinuation criteria

The DevelopAKUre consortium may discontinue the study prior to inclusion of the intended number of patients, but only in the event that new scientific information which could undermine the validity of the study, jeopardize patient safety or renders the study unethical.

After such a decision, the investigator must contact all participating patients as soon as possible. All study materials must be collected and all the CRFs completed to the greatest extent possible.

13 Dissemination and publication of results

The DevelopAKUre consortium will publicly register this study by posting the protocol on www.clinicaltrials.gov.

Following completion of the study, the results will be disseminated in line with the FP7 Agreement and will include publication in reputable peer reviewed medical journals.

The DevelopAKUre consortium will be responsible for these activities and will work with the investigators to determine how the publication is written, the number and order of authors, the journal(s) / scientific meeting(s) to which it will be submitted, and other related issues. In order to safeguard the potential for publication in a high-impact medical journal, the results of the study, or any part thereof, shall not be published by any person or organization outside the DevelopAKUre consortium without the prior written approval of the consortium, such consent and approval not to be unreasonably withheld.

14 Reference list

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Sensitivity/tipping point analysis