TITLE

Nitisinone arrests but does not reverse ochronosis in alkaptonuric mice

Craig M Keenan*, Andrew J Preston*, Hazel Sutherland, Peter J Wilson, Eftychia E Psarelli, Trevor F Cox, Lakshminarayan R Ranganath, Jonathan C Jarvis, James A Gallagher. *CMK and AJP contributed equally. JCJ and JAG contributed equally.

Craig M Keenan, Hazel Sutherland, Peter J Wilson, Lakshminarayan R Ranganath, James A Gallagher#

Department of Musculoskeletal Biology, Institute of Ageing and Chronic Disease, University of Liverpool, Sherrington Building, Ashton Street, Liverpool, L69 3GE, UK

#Email: jag1@liverpool.ac.uk – Corresponding author

Andrew J Preston

Developmental Immunology, Paediatrics, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DS, UK

Eftychia E Psarelli, Trevor F Cox

Cancer Research UK Liverpool Cancer Trials Unit, University of Liverpool, 1-3 Brownlow Street, L69 3GL, UK

Lakshminarayan R Ranganath

Department of Clinical Biochemistry and Metabolism, Royal Liverpool University Hospital, Prescot Street, Liverpool, L7 8XP, UK

Hazel Sutherland, Jonathan C Jarvis

School of Sport and Exercise Sciences, Liverpool John Moores University, Tom Reilly Buildings, Byrom Street, Liverpool, L3 3AF, UK

Text word count: 1821

Summary word count: 224

Number of figures: 4

SUMMARY

Alkaptonuria (AKU) is an ultra-rare autosomal recessive disorder resulting from a deficiency of homogentisate 1,2 dioxygenase (HGD), an enzyme involved in the catabolism of phenylalanine and tyrosine. Loss of HGD function prevents metabolism of homogentisic acid (HGA) leading to increased levels of plasma HGA and urinary excretion. Excess HGA becomes deposited in collagenous tissues and subsequently undergoes polymerization, principally in the cartilages of loaded joints, in a process known as ochronosis. This results in an early onset, devastating osteoarthropathy for which there is currently no effective treatment. We recently described the natural history of ochronosis in a murine model of AKU, demonstrating that deposition of ochronotic pigment begins very early in life and accumulates with age. Using this model we were able to show that lifetime treatment with nitisinone, a potential therapy for AKU, was able to completely prevent deposition of pigment. However, although nitisinone has been shown to inhibit ochronotic deposition, whether it can also facilitate removal of existing pigment has not yet been examined. We describe here that mid-life administration of nitisinone to AKU mice arrests further deposition of ochronotic pigment in the tibio-femoral joint, but does not result in the clearance of existing pigment. We also demonstrate the dose-dependent response of plasma HGA to nitisinone, highlighting its efficacy for personalised medicine, where dosage can be tailored to the individual AKU patient.

SYNOPSIS

Nitisinone arrests further deposition of ochronotic pigment when administered mid-life, it does not reduce existing pigmentation, and it reduces the plasma HGA levels in a dose-dependent manner.

Compliance with Ethics Guidelines

CONFLICTS OF INTEREST

Craig M Keenan, Andrew J Preston, Hazel Sutherland, Peter J Wilson, Eftychia E Psarelli, Trevor F Cox, Lakshminarayan R Ranganath, Jonathan C Jarvis and James A Gallagher declare they have no conflict of interest.

ANIMAL RIGHTS

All institutional and national guidelines for the care and use of laboratory animals were followed.

AUTHOR CONTRIBUTIONS

CMK and AJP were involved in data acquisition, analysis and reporting of the work.
HS and PJW were involved in data acquisition.
EEP and TFC were involved data analysis.
LRR, JCJ and JAG were involved in the planning of the work.

INTRODUCTION

Alkaptonuria (AKU) has a unique place in the history of metabolic disease as the first disorder to be described as an 'inborn error of metabolism' by the distinguished English physician, Sir Archibald Garrod (Garrod 1902). AKU is an ultra-rare autosomal recessive disorder with a worldwide incidence of between 1 in 250,000-1,000,000 live births (Phornphutkul et al 2002). AKU results from mutations in homogentisate 1,2-dioxygenase (HGD) (EC 1.13.11.5), the enzyme involved in the catabolism of phenylalanine and tyrosine (La Du et al 1958). Loss of HGD function results in both increased plasma levels of homogentisic acid (HGA), and HGA excretion. Urinary HGA darkens on exposure to air, and is typically observed as the first symptom in patients who present with AKU. Elevated levels of plasma HGA ultimately leads to the pigmentation of cartilaginous tissues, following the deposition and subsequent polymerisation of HGA in a process known as ochronosis. This results in an early-onset, devastating osteoarthropathy for which there is currently no effective treatment.

Nitisinone (2-(2-nitro-4-(trifluoromethyl) benzoyl) cyclohexane-1,3-dione) is a reversible inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPPD) (EC 1.13.11.27), the enzyme responsible for producing HGA. Originally developed as a herbicide (Schulz et al 1993), it is routinely used for the treatment of hereditary tyrosinaemia type 1 (McKiernan 2006). Nitisinone is viewed as a potential treatment for AKU as it prevents accumulation of HGA in plasma. Ochronosis has recently been described in two murine models of AKU (Taylor et al 2012, Preston et al 2014). In the latter of the two models, we have described the efficacy of nitisinone in treating ochronosis in a murine model of AKU and demonstrated that lifetime administration of nitisinone reduced plasma HGA by 88% and prevented ochronotic pigment deposition in the tibio-femoral joint (Preston et al 2014). This was the first time that inhibition of ochronosis by nitisinone had been demonstrated, and highlighted the efficacy of nitisinone as a treatment for AKU.

As a large proportion of AKU patients already suffer from osteoarthropathy, it is important to determine if nitisinone's efficacy is purely prophylactic, or whether it can facilitate repair and regeneration of damaged cartilage during natural metabolic turnover. Although pigmentation in AKU mice can be prevented throughout their lifetime by administration of

nitisinone, there is no data on whether ochronosis is reversible. Here we describe that the mid-life administration of nitisinone to AKU mice successfully arrests further deposition of ochronotic pigment in the tibio-femoral joint, but does not result in the reduction of existing pigment. We also demonstrate that the response of plasma HGA to nitisinone treatment is dose-dependent, which should facilitate tailored treatment of AKU patients presenting with differing degrees of severity.

MATERIALS AND METHODS

Mice

Hgd-/- (AKU) mice on a BALB/c or C57BL/6 background were used for all experiments. All mice were housed and maintained within the University of Liverpool's Biological Services Unit (BSU) in accordance with Home Office UK guidelines.

Sample preparation

Tail bleed samples were collected into microvettes (Sarstedt, CB 300) and stored at 4°C prior to processing within 2hrs, using an adaptation of the Bory method (Bory et al 1990). Briefly, whole blood was centrifuged at 1500×g for 10 min at 4°C, and the plasma deproteinised by adding 5.8 M perchloric acid (Sigma, UK) equivalent to 10% of the plasma and containing 0.1 mM 4-amino-2-chlorobenzoic acid (Sigma, UK) as internal standard. Acidified supernatant was stored at −20°C. A 150µl tail-bleed volume yielded approximately 25µl of deproteinised plasma.

Chromatographic conditions

Plasma HGA concentration was determined via HPLC as described previously (Preston et al 2014), on a Phenomenex Kinetex XB-C18 column, 2.6µ (4.6 x100mm). Briefly, the initial mobile phase was 100% buffer A (12mM orthophosphoric acid, Sigma, UK), before increasing buffer B (100% methanol, Sigma, UK) from 0-80% over 10mins. Detection was by UV at 290nm.

Mid-life nitisinone treatment

A cohort of eight BALB/c Hgd-/- mice (four male, four female) were provided with filtered water from 8 to 34 weeks of age. They were then provided with an ad libitum supply of water containing 4mg/l of nitisinone (Shanghai Elittes Organics, China) from 34 to 79 weeks of age. The control group of 8 BALB/c Hgd-/- mice (four male, four female) were untreated over the same time period. Plasma was taken at 35 weeks, and then sampled regularly by tail bleed over the mouse lifetime. Tibio-femoral joints were taken for histological analysis at end of study. Analysis of joint pigmentation was also performed at different ages, in either BALB/c Hgd-/- or C57BL/6 Hgd-/- mice to build up a disease progression timeline.

Nitisinone dose-response

Six cohorts of four age-matched C57BL/6 Hgd-/- mice (two male, two female) had their plasma sampled at 54 weeks, and then immediately treated with an ad libitum supply of water containing either 4mg/L, 1mg/L, 0.5mg/L, 0.25mg/L, 0.125mg/L, or 0mg/L of nitisinone for 13 days. Plasma was sampled again 7 and 19 days post treatment, and its HGA concentration determined by HPLC.

Histological analysis

Mice were euthanised with Pentoject (sodium pentobarbitone 20% w/v) and their tibio-femoral joint harvested and stored in 10% phosphate buffered formalin solution, pH 7.4, for a minimum of 24hrs. Tissues were washed in phosphate buffered saline before decalcification in 12% EDTA for 7 days. Tibio-femoral joints were dissected free of excess muscle then paraffin embedded in the coronal plane to enable simultaneous evaluation of both the medial and lateral compartments of the joint, as recommended by the Osteoarthritis Research Society International (OARSI) histopathology initiative. The first section that encompassed both the tibial plateau and femoral condyles was selected as representative of each mouse. Sections were mounted on glass slides, rehydrated and stained with H&E or Schmorl's stain, previously shown to be a sensitive method for the detection of ochronotic pigment (Tinti, Taylor et al. 2011). Sections were dehydrated through graded alcohols, and mounted with DPX resin (VWR International, UK) for examination by light microscopy.

Quantification of pigmented chondrons

The first whole section that encompassed the entire tibio-femoral joint (MTP, MFC, LTP, & LFC) was selected as representative of each mouse for quantification analysis. From these sections, pigmented chondrons present in the articular cartilage and entheses of the femoral condyles and articular cartilage of the tibial plateau were quantified.

Statistical analyses

Comparisons in pigmentation rates between treated and untreated groups were performed using an independent samples t-test. Descriptive statistics are reported as mean and standard deviation (SD) as normality was achieved, while results were considered as

statistically significant at the 5% level. Data analysis was undertaken using Stata 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP.).

RESULTS

Mid-life treatment with 4mg/L nitisinone from 35-79 weeks of age suppressed plasma HGA concentration by approximately fifteen fold, in agreement with previous work (Preston et al 2014) (Fig. 1). The reduction in plasma HGA following 44 weeks of nitisinone treatment, translated into a statistically significant difference (t=4.645, *p*=0.001) in the mean number of pigmented chondrons visible in the nitisinone treated mice at 79 weeks, equal to 88 (SD=26.5) (Fig. 2b), relative to aged-matched untreated AKU mice, equal to 201.2 (SD=48.4) (Fig. 3). The degree of pigmentation observed in nitisinone treated BALB/c Hgd-/- mice at 79 weeks was considered equivalent to that observed in untreated 34 week old BALB/c Hgd-/- mice (Fig. 2a), correspondent with the time at which treatment began. This demonstrated that mid-life treatment with nitisinone arrested further deposition of ochronotic pigment but did not clear previously laid-down pigment, resulting in higher observable chondron pigmentation than AKU mice treated from birth (Fig. 2d). The number of pigmented chondrons observed in the untreated mice at 79 weeks (Fig. 2c) was consistent with levels previously reported in the natural history study of BALB/c Hgd-/- mice (Preston et al 2014).

Quantification of pigmented chondrons over time (Fig. 3) confirmed that although the degree of deposition between AKU mice was highly variable (even within highly inbred mouse strains); progressive accumulation of ochronotic pigment was consistent with ageing. Nevertheless, mid-life treatment with nitisinone effectively inhibited further deposition of ochronotic pigment.

Plasma HGA concentration was also highly variable in AKU mice, but was observed to respond in a highly dose-dependent fashion to nitisinone treatment, when plotted as a percentage of its pre-treatment value (Fig. 4). Higher concentrations of nitisinone resulted in greater suppression of plasma HGA variability within cohorts, while removal of nitisinone after 13 days resulted in a rebound of plasma HGA levels.

DISCUSSION

We have previously shown that nitisinone treatment from birth can prevent ochronosis in the adult AKU mouse (Preston et al 2014). Here we demonstrate that beginning nitisinone treatment mid-way through life (34 weeks) is sufficient to arrest further disease progression. Remarkably, 45 weeks after treatment, the mean number of pigmented chondrons observed within the knee joint was no greater than that typical of untreated 34 week old AKU mice, according to our disease progression timeline (Fig. 3). Unlike in mice treated with nitisinone from birth however (Fig. 2d), pigmentation could still be observed and quantified. It is evident therefore, that while nitisinone can prevent further deposition of ochronotic pigment, it does not reduce pre-existing pigmentation by enabling turnover/replacement of damaged cartilage. This strongly implies that in order to minimise the irreparable joint damage typical of AKU disease progression in humans, treatment with nitisinone should begin as early as possible. Although there was no evidence that mid-life treatment with nitisinone could facilitate removal of existing pigmentation, it did arrest any further deposition of ochronotic pigment which may lead to the prevention or slowing down of disease progression in patients with established ochronosis.

Establishing a minimum effective nitisinone dose is fundamental to reducing the cost of lifetime treatment and minimising potential side effects such as corneal keratopathy (Introne et al 2011). As plasma HGA concentration in AKU patients is highly variable, we therefore examined dose–response sensitivity to nitisinone to determine the practicality of tailoring nitisinone treatment dose to the patient. A clear dose-response effect was observed between nitisinone and plasma HGA levels, which decreased consistently following increased doses of nitisinone. Treatment with 4mg/L nitisinone reduced plasma HGA by 90% over a 13-day period when compared with baseline controls. Ranganath and colleagues recently showed a similar dose-response effect of nitisinone when analysing urinary HGA excretion over a 24h period in AKU patients (Ranganath et al 2014). Both our data and that of Ranganath et al highlight the efficacy of nitisinone in reducing the levels of circulating HGA. As excellent dose-response sensitivity was observed between differing nitisinone concentrations and plasma HGA levels, it may be possible to tailor individual treatment plans for AKU patients.

In summary we have shown that nitisinone can effectively inhibit ochronotic deposition in an alkaptonuric mouse model, and if introduced mid-life can arrest any further disease progression. Nitisinone treatment does not result in the removal of existing ochronotic pigmentation, and cannot therefore be used to treat existing joint damage. Plasma HGA concentrations display excellent sensitivity to treatment dose, facilitating the tailoring of therapy to patient disease severity.

REFERENCES

Bory C, Boulieu R, Chantin C, Mathieu M (1990). "Diagnosis of alcaptonuria: rapid analysis of homogentisic acid by HPLC." Clin Chim Acta **189**(1): 7-11.

Garrod AE (2002). "The incidence of alkaptonuria: a study in chemical individuality. 1902 [classical article]." Yale J Biol Med **75**(4): 221-231.

Introne WJ, Perry MB, Troendle J et al (2011). "A 3-year randomized therapeutic trial of nitisinone in alkaptonuria." Mol Genet Metab **103**(4): 307-314.

La Du BN, Zannoni VG, Laster L, Seegmiller JE (1958). "The nature of the defect in tyrosine metabolism in alcaptonuria." J Biol Chem **230**(1): 251-260.

McKiernan, P. J. (2006). "Nitisinone in the treatment of hereditary tyrosinaemia type 1." Drugs **66**(6): 743-750.

Phornphutkul C, Introne WJ, Perry MB et al (2002). "Natural history of alkaptonuria." N Engl J Med **347**(26): 2111-2121.

Preston AJ, Keenan CM, Sutherland H et al (2014). "Ochronotic osteoarthropathy in a mouse model of alkaptonuria, and its inhibition by nitisinone." Ann Rheum Dis **73**(1): 284-289.

Ranganath LR, Milan AM, Hughes AT et al (2014). "Suitability Of Nitisinone In Alkaptonuria 1 (SONIA 1): an international, multicentre, randomised, open-label, no-treatment controlled, parallel-group, dose-response study to investigate the effect of once daily nitisinone on 24-h urinary homogentisic acid excretion in patients with alakptonuria after 4 weeks of treatment." Ann Rheum Dis(0): 1-7.

Schulz A, Ort O, Beyer P, Kleinig H (1993). "SC-0051, a 2-benzoyl-cyclohexane-1,3-dione bleaching herbicide, is a potent inhibitor of the enzyme p-hydroxyphenylpyruvate dioxygenase." FEBS Lett **318**(2): 162-166.

Taylor AM, Preston AJ, Paulk NK et al (2012). "Ochronosis in a murine model of alkaptonuria is synonymous to that in the human condition." Osteoarthritis Cartilage **20**(8): 880-886.

Tinti L, Taylor AM, Santucci A et al (2011). "Development of an in vitro model to investigate joint ochronosis in alkaptonuria." Rheumatology (Oxford) **50**(2): 271-277.

FIGURES

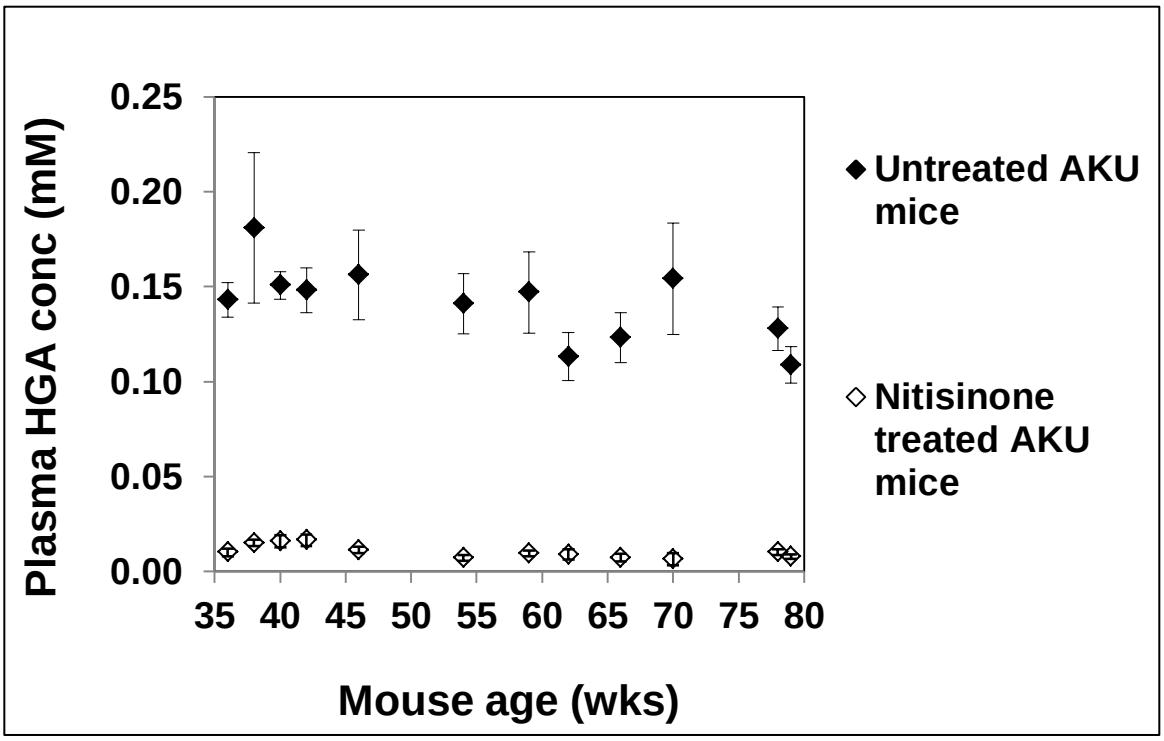

Fig. 1 The effect of mid-life (34 weeks) dietary supplementation with 4mg/L nitisinone on the plasma HGA concentration in AKU mice.

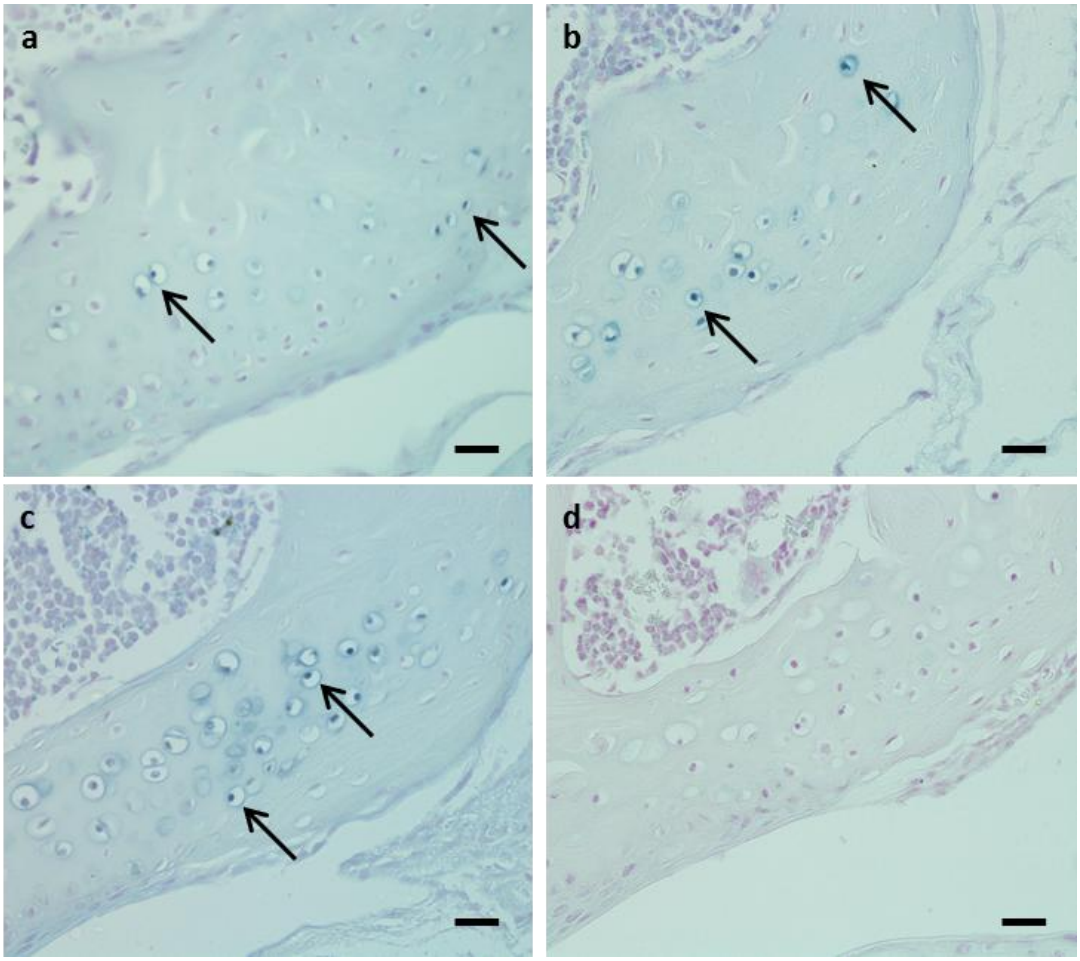

Fig. 2 a Photomicrograph displaying pigmented chondrons (arrows) in a 35 week old BALB/c Hgd-/- mouse, prior to treatment with nitisinone. **b** Administration of nitisinone (4mg/l) at 35 weeks prevented large scale pigmentation of chondrons in the tibio-femoral joint. The number of pigmented chondrons (arrows) observed in the nitisinone treated BALB/c Hgd-/- mice at 80 weeks was comparable to those seen in untreated BALB/c Hgd-/- mice at 35 weeks (Fig.1, 2a). **c** Photomicrograph of an 80 week old untreated BALB/c Hgd-/- mouse. Large numbers of pigmented chondrons (arrows) were present throughout the tibio-femoral joint, highlighting the effectiveness of nitisinone when given mid-life (Fig. 2b). **d** Lifetime treatment with nitisinone (4mg/l) prevented any deposition of ochronotic pigment in the tibio-femoral joint. All images were taken from the lateral femoral condyle of BALB/c Hgd-/- mice. Bar = 20µm.

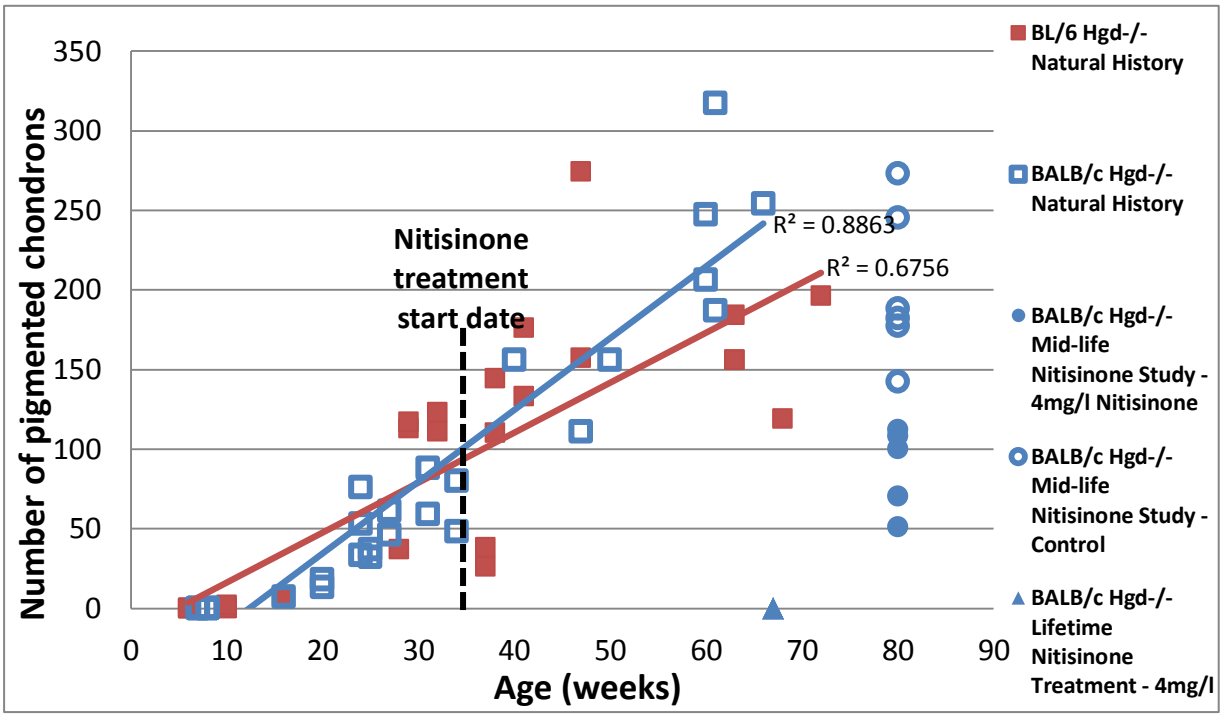

Fig. 3 Quantification of pigmented chondrons in the tibio-femoral joint of AKU mice, depicting ochronotic pigment deposition over time, and the effect of treatment with 4mg/L nitisinone when administered mid-life. Treatment at 34 weeks prevented further deposition of ochronotic pigment by week 79, but did not reverse the effects of previously laid down pigment. Quantification of pigmented chondrons was performed on a single section from each mouse, and does not represent the total cell number in each mouse.

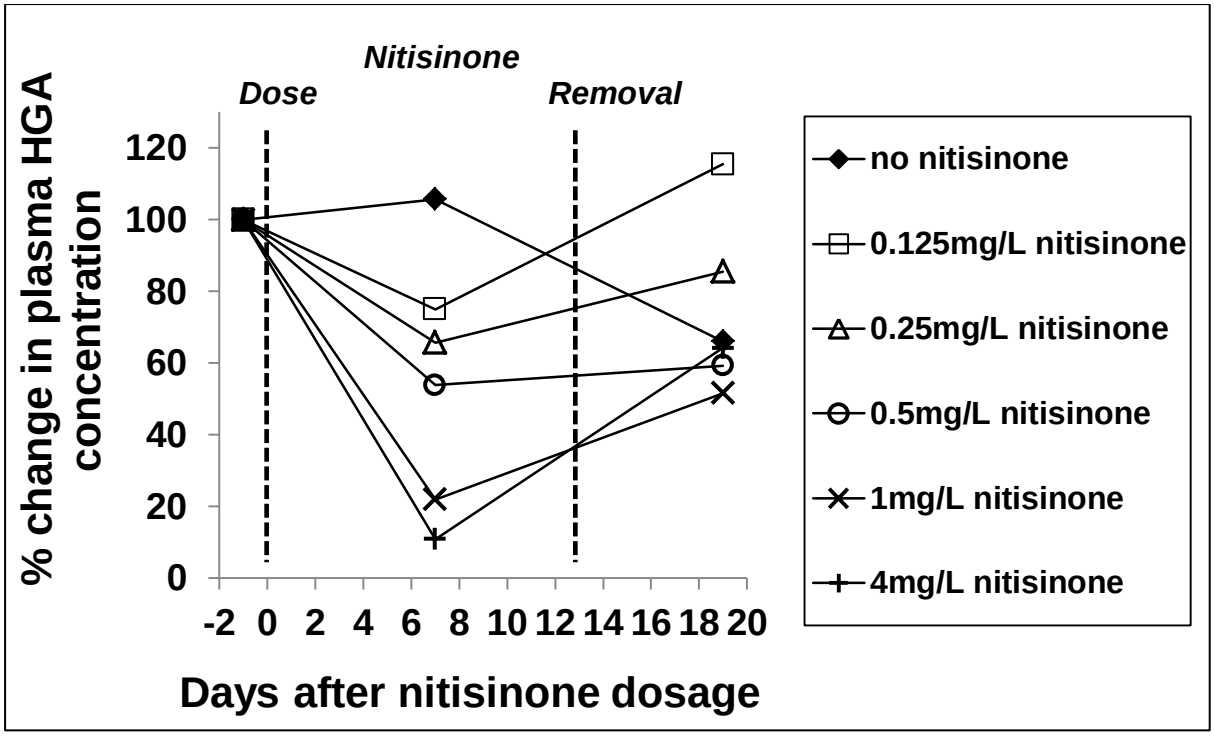

Fig. 4 The dose response to nitisinone (as percentage change of plasma HGA concentration) in AKU mice, and recovery.

Figure 1
Click here to download high resolution image

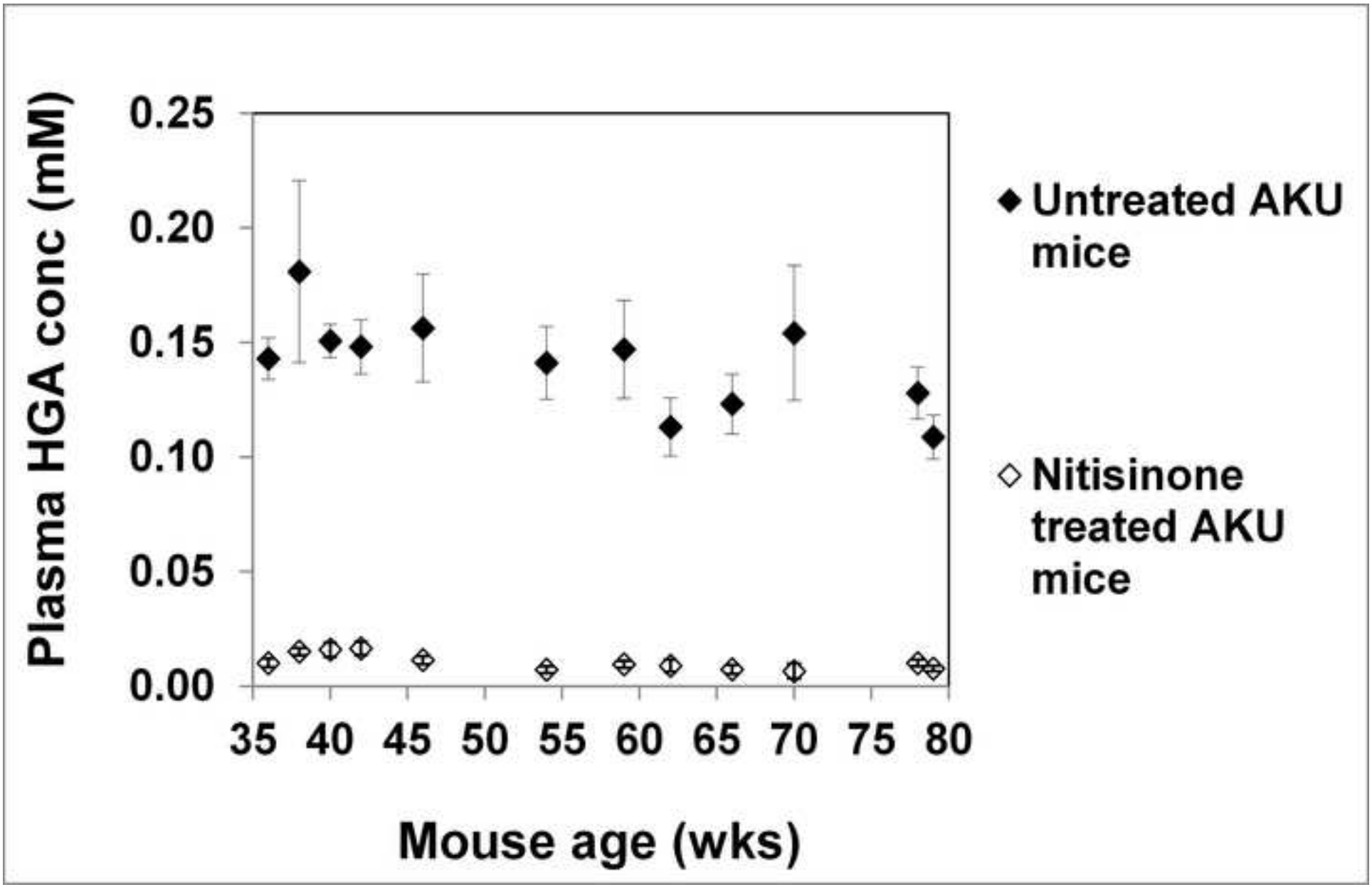

Figure 2a
Click here to download high resolution image

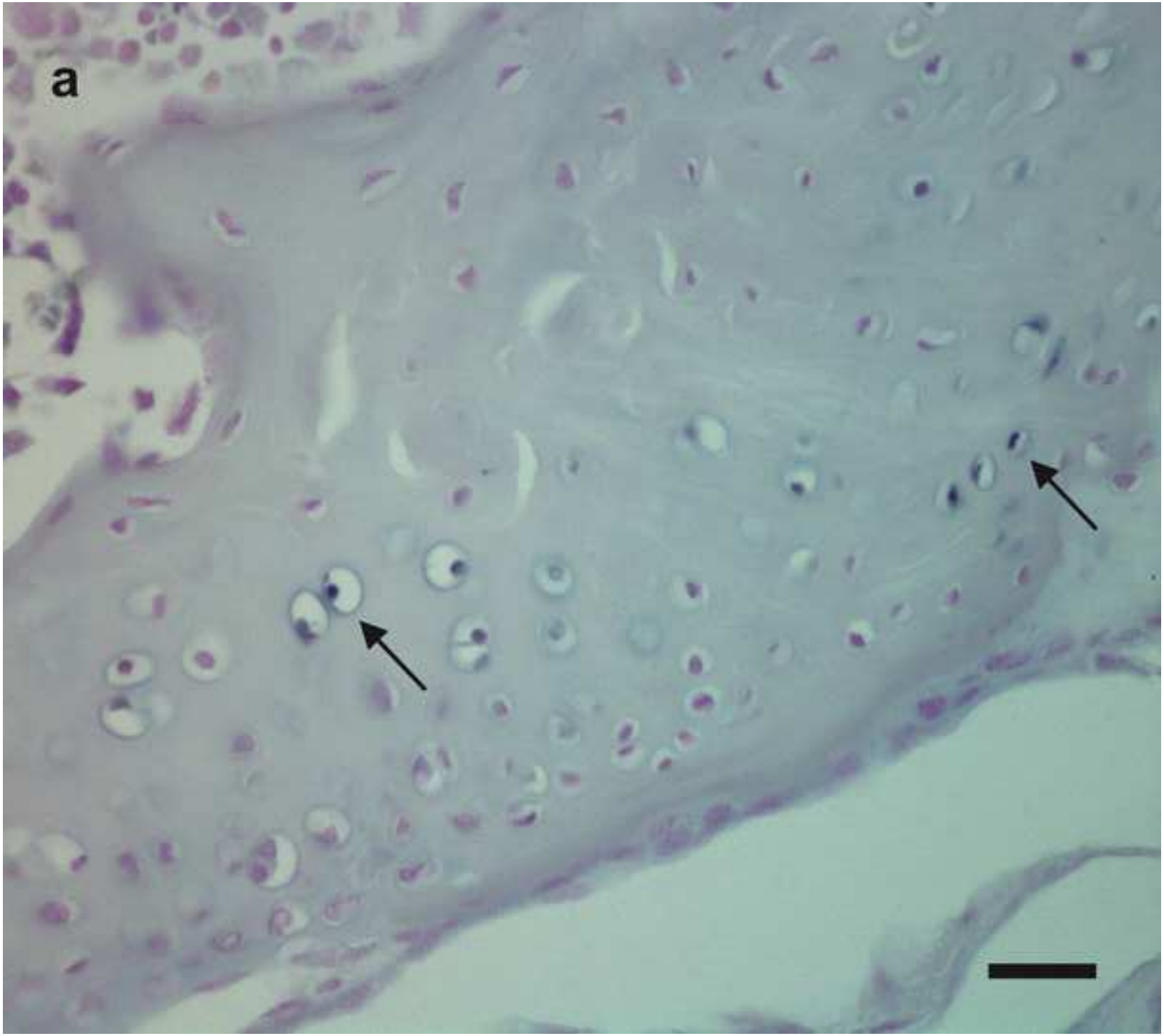

Figure 2b
Click here to download high resolution image

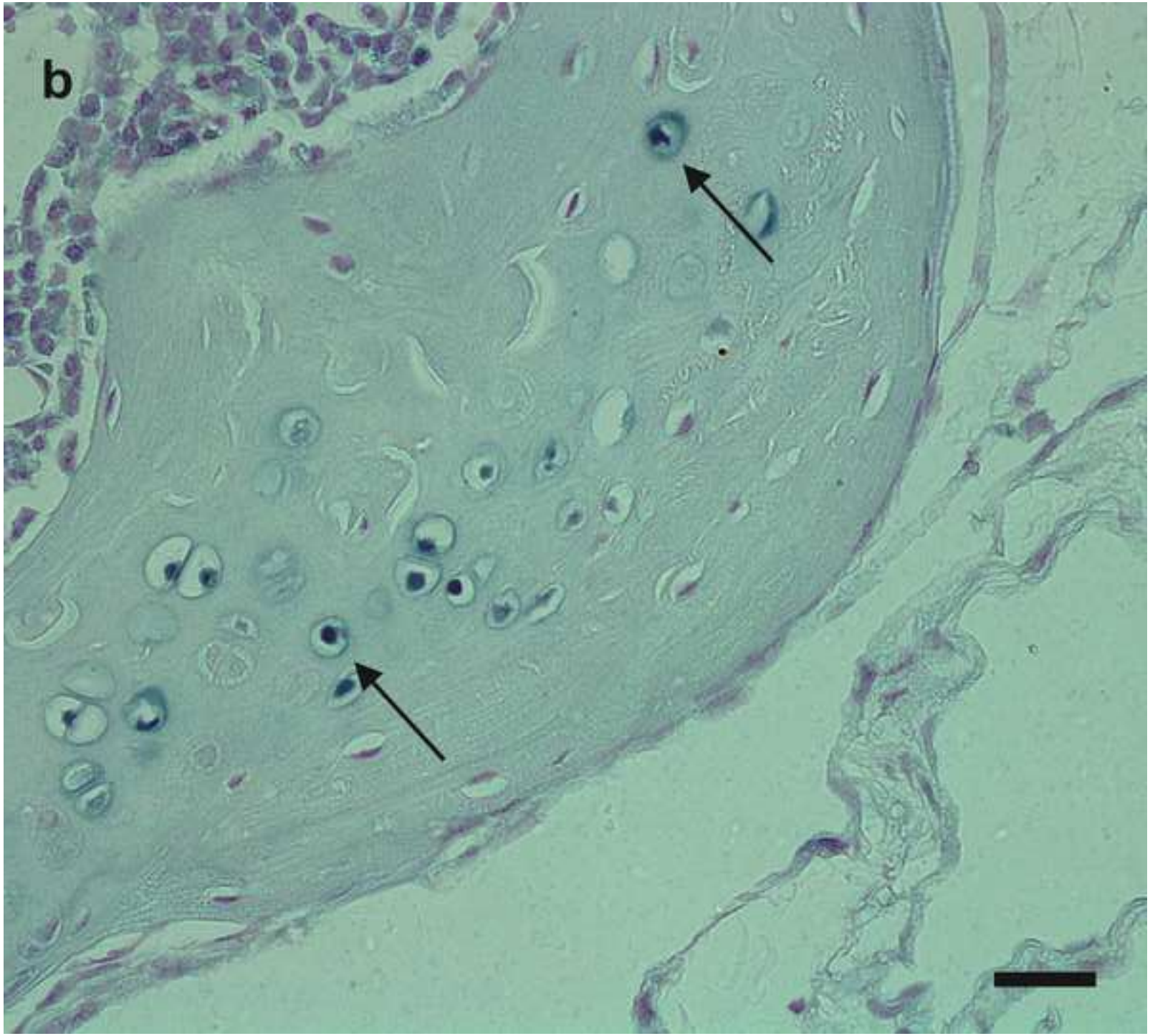

Figure 2c
Click here to download high resolution image

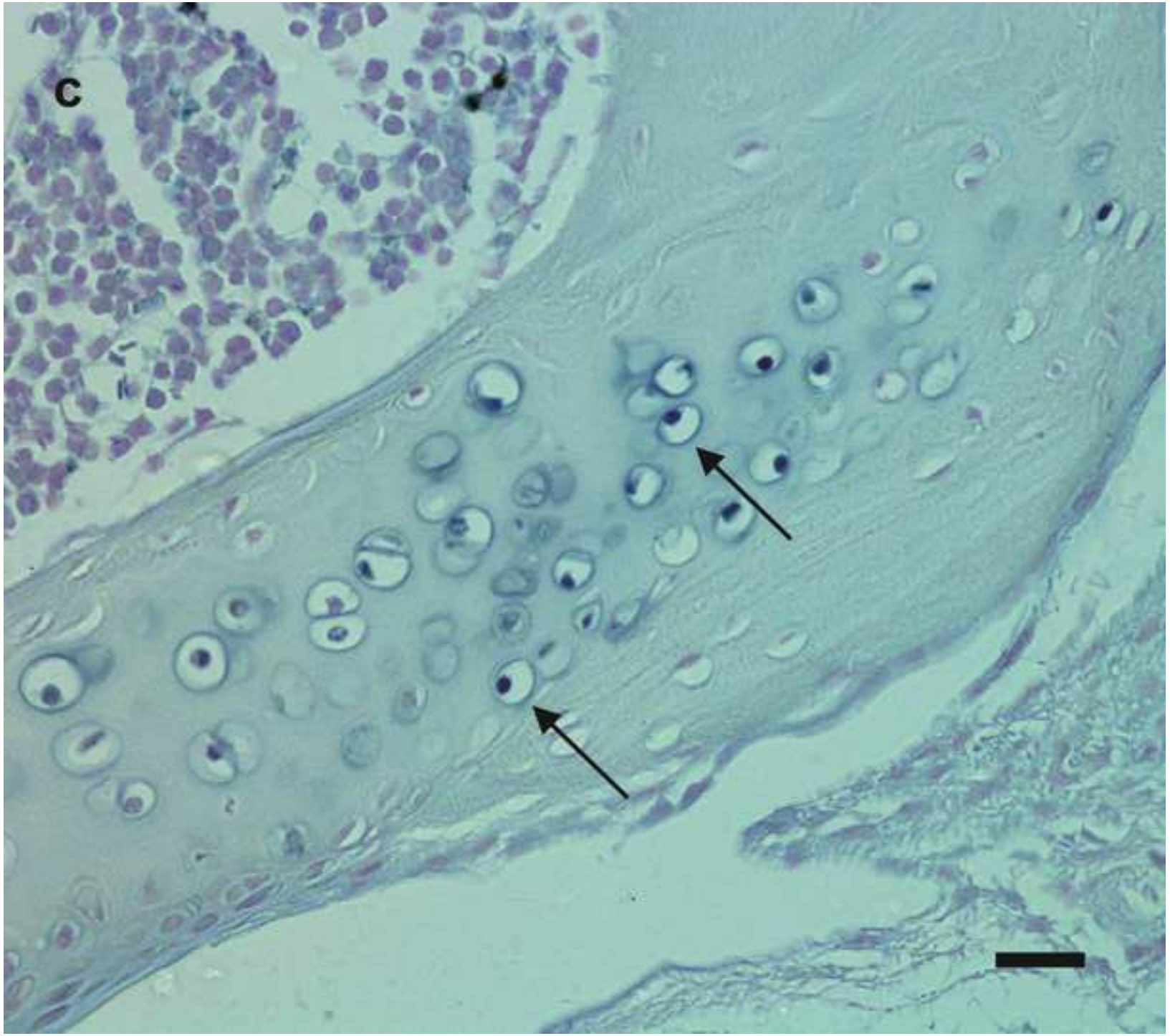

Figure 2d
Click here to download high resolution image

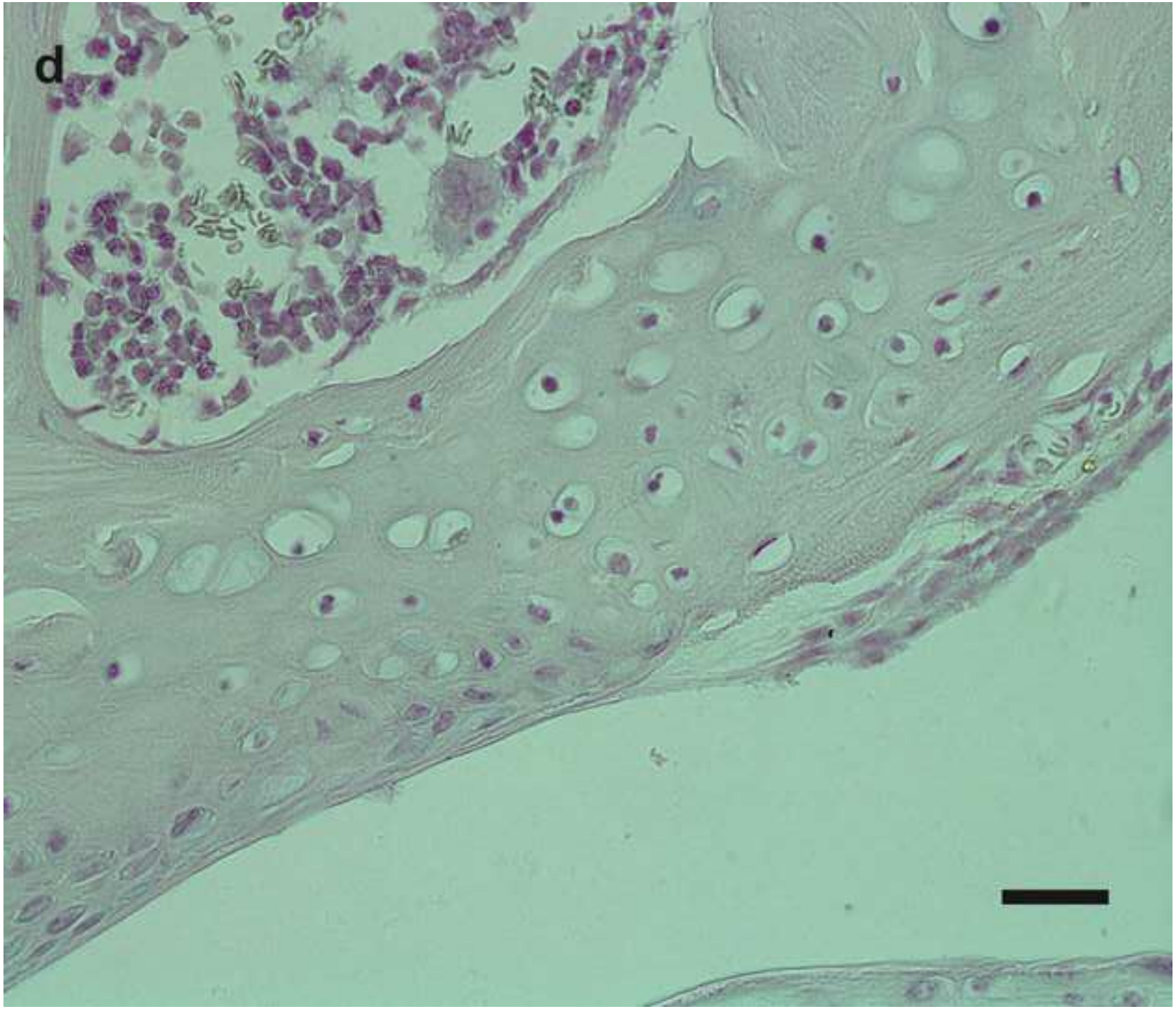

Figure 3
Click here to download high resolution image

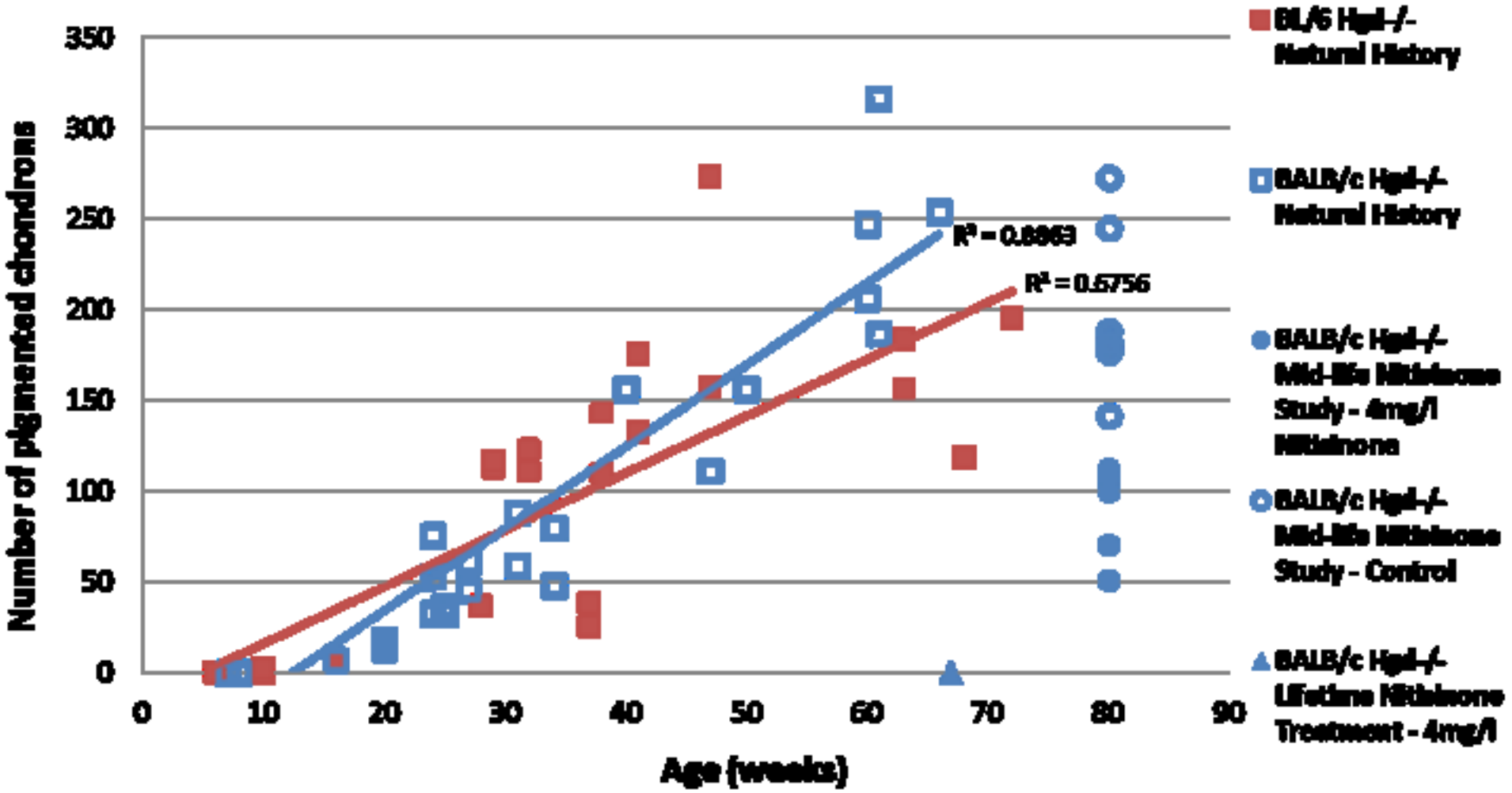

Figure 4
Click here to download high resolution image