LETTER

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Lanosterol reverses protein aggregation in cataracts

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The human lens is comprised largely of crystallin proteins assembled into a highly ordered, interactive macro-structure essential for lens transparency and refractive index. Any disruption of intra- or inter-protein interactions will alter this delicate structure, exposing hydrophobic surfaces, with consequent protein aggregation and cataract formation. Cataracts are the most common cause of blindness worldwide, affecting tens of millions of people[1], and currently the only treatment is surgical removal of cataractous lenses. The precise mechanisms by which lens proteins both prevent aggregation and maintain lens transparency are largely unknown. Lanosterol is an amphipathic molecule enriched in the lens. It is synthesized by lanosterol synthase (LSS) in a key cyclization reaction of a cholesterol synthesis pathway. Here we identify two distinct homozygous *LSS* missense mutations (W581R and G588S) in two families with extensive congenital cataracts. Both of these mutations affect highly conserved amino acid residues and impair key catalytic functions of LSS. Engineered expression of wild-type, but not mutant, *LSS* prevents intracellular protein aggregation of various cataract-causing mutant crystallins. Treatment by lanosterol, but not cholesterol, significantly decreased preformed protein aggregates both *in vitro* and in cell-transfection experiments. We further show that lanosterol treatment could reduce cataract severity and increase transparency in dissected rabbit cataractous lenses *in vitro* and cataract severity *in vivo* in dogs. Our study identifies lanosterol as a key molecule in the prevention of lens protein aggregation and points to a novel strategy for cataract prevention and treatment.

Cataracts account for over half of all cases of blindness worldwide, with the only established treatment involving surgical removal of the opacified lens. In developed nations, cataract surgeries amount to a significant portion of healthcare costs owing to the sheer prevalence of the disease among ageing populations. In addition, there is major morbidity associated with cataracts in developing countries, where there is limited access to surgical care.

High concentrations of crystallin proteins in lens fibres contribute to lens transparency and refractive properties[2]. The crystallin superfamily is composed of α-, β- and γ-crystallins, which are some of the most highly concentrated intracellular proteins in the human body. Protein aggregation is the single most important factor in cataract formation[3]. Factors that lead to protein aggregation include mutations in crystallin proteins, which are known to cause congenital cataracts, or oxidative stress, which in turn contributes to age-related cataracts. However, the precise mechanisms by which lens proteins maintain transparency or cause opacification are not completely understood.

Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase, LSS; EC 5.4.99.7) is encoded by the *LSS* gene. The LSS protein catalyses the conversion of (*S*)-2,3-oxidosqualene to lanosterol, which is a key early rate-limiting step in the biosynthesis of cholesterol, steroid hormones, and vitamin D (ref. 4). LSS was found to be expressed in the lens[5]. It was previously reported that the specific combination of hypomorphic mutations on *LSS* and *FDFT1* (farnesyl diphosphate farnesyl transferase 1) could decrease cholesterol levels in the lens and result in cataracts in Shumiya cataract rats (SCR)[6]. Here we identify novel homozygous mutations in the *LSS* gene in two consanguineous families and investigate the ability of lanosterol to alleviate protein aggregation and cataract formation.

We identified three children with severe congenital cataract from a consanguineous family of Caucasian descent (Fig. 1a). We performed whole-exome sequencing to an average of no less than 55-fold depth coverage on the target region (Extended Data Table 1a) in order to identify the causal mutation. On average, ~60,800–80,800 SNPs were detected in each exome (Extended Data Table 1b). Using a consanguineous recessive model and filtering against common variants (minor allele frequency >0.5%) in public databases, including dbSNP and the 1000 Genomes Project, as well as mutation function predictions (predicted by SIFT[7], Polyphen2[8], Phylop[9] and Mutationtaster[10]), we narrowed down potential candidate gene variants and identified a variant (G588S) in *LSS* on chromosome 21 as the most likely candidate (Extended Data Table 1c). Three affected children were homozygous for the G→A transition (G588S) in *LSS*, (GRch37/hg19: chr21:47615645; NM_001001438.2:c.1762G > A, NM_001001438.1: p.G588S), while the unaffected father, mother and remaining child were heterozygous for the change (Fig. 1a, b). Whole-genome SNP genotyping identified three long continuous homozygous regions in this family by HomozygosityMapper[11] (chr2:q22.1–q24.1, chr2:q31.1–q32.1 and chr21:q22.3; Extended Data Fig. 1a and Extended Data Table 1d). The *LSS* gene was located in one of the homozygous regions on chromosome 21 (Extended Data Fig. 1b). Furthermore, we screened for mutations in the *LSS* gene in 154 families with congenital cataracts and identified another homozygous mutation, W581R (GRch37/hg19: chr21:47615666; NM_001001438.2:c.1741T > C, NM_001001438.1: p.W581R), in a second consanguineous family (Fig. 1a, b, c). These two mutations were absent in 11,000 control chromosomes.

The amino acid residues W581 and G588 in LSS are highly conserved (Fig. 2a). We performed computational modelling analysis to

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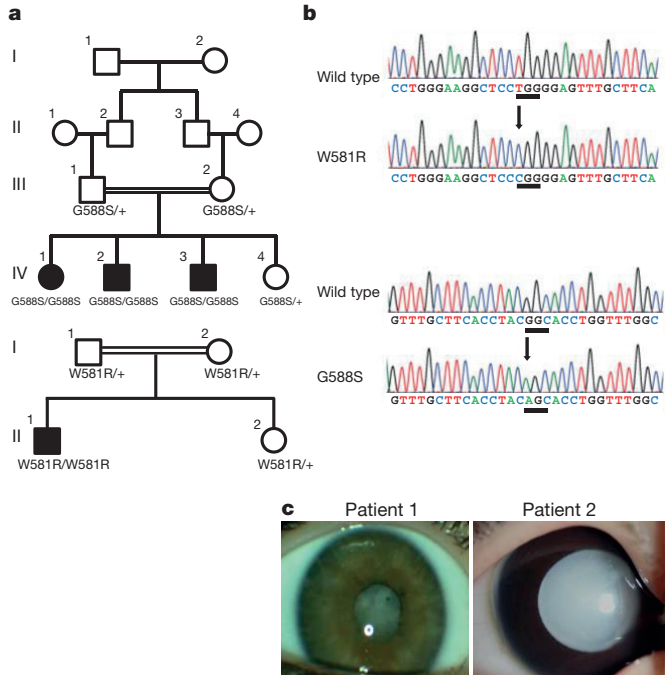

Figure 1 | Identification of mutations in *LSS* causing congenital cataracts. **a**, Pedigrees of affected families and cataract phenotype. Squares and circles indicate males and females respectively. +, wild-type allele; W581R and G588S are the two mutations. **b**, Upper panel, DNA sequencing data of an unaffected individual and an affected child (II-1) with a homozygous W581R mutation; lower panel, DNA sequencing data of an unaffected individual and an affected child (IV-1) with a homozygous G588S mutation. The underlined sequence indicates the nucleic acid change. **c**, Left, colour photograph of patient 1's right eye in the first pedigree (IV-1) with a total cataract; right, colour photograph of patient 2's right eye in the same pedigree (IV-3) with a cataract.

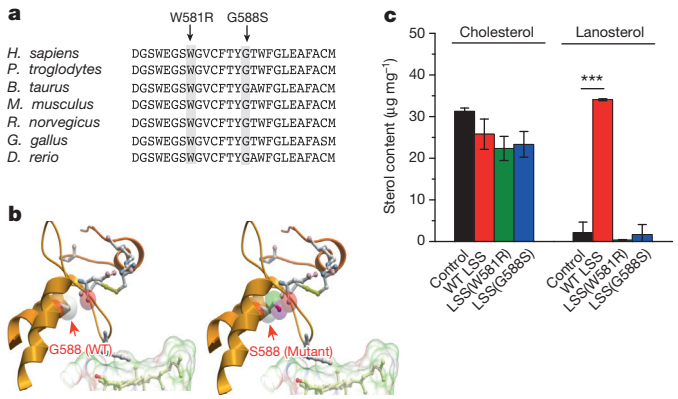

Figure 2 | LSS mutations abolished the cyclase enzymatic function. **a**, Conservation of W581R and G588 in LSS across several species: *Homo sapiens*, *Pan troglodytes*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus* and *Danio rerio*. **b**, Computer modelling of LSS structure and impact of the LSS W581R and G588S mutations. A computer modelling analysis identifies a loop originating from C584 and ending at E578 with the key side chain of W581 at the tip of the loop stabilizing the sterol. The loop is fixed by an S–S bridge and the E578–R639 salt bridge. Amide nitrogen N of G588 interacts with the C584 from the previous helical turn and the Cα hydrogen of G588 is in close proximity to the critical E578, which then forms a strong salt bridge with R639 of the same supporting helix. The mutation G588S causes the side chain of the serine to clash into the E578 residue of the loop and is incompatible with the structure. Arrow indicates the location of the mutant side chain. **c**, Effect of engineered expression of the wild-type protein (WT LSS) and LSS mutants on sterol content. Wild-type LSS markedly increased lanosterol production, whereas neither W581R nor the G588S mutant exhibited any cyclase activity. $n = 3$ in each group; $^{***}P < 0.001$.

investigate the effects of the W581R and G588S mutations on the 3D structure and function of LSS. The amino acid tryptophan at position 581 has been reported to contribute to the catalytic site of the cyclase activity[12]. The G588S mutant was modelled by in-place replacement followed by side-chain refinement. The S588 side-chain refinement could not resolve the van der Waals clash between the serine side chain and the backbone carbonyl of E578, which forms a key salt bridge with R639. The orientation of the E579:C584 loop needed to be distorted to accommodate the mutation. The side chain of the mutant S588 clashed into an adjacent loop, indicating that the mutation was incompatible with the normal enzymatic structure and function of LSS (Fig. 2b). Supporting the *in silico* results, expression of wild-type LSS in a cell-transfection experiment exhibited cyclase activity and dramatically increased the amount of lanosterol production in the lipid fraction in HeLa cells, while neither the W581R nor the G588S mutant protein demonstrated any cyclase activity (Fig. 2c).

In contrast, the cholesterol level was unaffected by the expression of wild-type or mutant LSS, suggesting that there may be an alternative pathway for cholesterol homeostasis. The W581R and G588S mutations did not alter subcellular localization or cause aggregates of LSS protein when compared to that of wild-type LSS, suggesting that the cataract phenotype was not due to the formation of light-scattering particles by mutant LSS proteins themselves (Extended Data Fig. 2).

The aggregation of crystallins, the major structural proteins in the lens, is a predominant cause of various types of cataracts[3]. To mimic protein aggregation in the cataractous lens, six known cataract-causing mutant crystallin proteins were expressed in human lens progenitor cells, human lens epithelial line B-3 (HLEB-3), or HeLa cells. These mutant crystallins formed p62-positive inclusion bodies/aggresomes in all three transfected cell lines, suggesting that aggregation is an intrinsic property of mutant crystallins (Fig. 3a and Extended Data Figs 3 and 4)[13]. Co-expression of wild-type LSS and a cataract-causing mutant crystallin protein significantly reduced both the number and size of intracellular crystallin aggregates, whereas LSS mutants failed to do so alone (Fig. 3b, c and Extended Data Figs 3 and 4). Western blot analysis indicated that the Y118D mutant of αA-crystallin was released from intracellular aggregates and became more soluble with wild-type LSS (Fig. 3d and Extended Data Fig. 4c). Furthermore, addition of lanosterol, but not cholesterol, in the culture medium of cells co-expressing an LSS mutant and a mutant crystallin successfully reduced crystallin aggregation (Fig. 3c and Extended Data Figs 3 and 4). This result indicated that lanosterol, but not cholesterol, could be an effective agent to release mutant crystallin proteins from aggregation. Supporting this hypothesis, lanosterol significantly inhibited aggresome formation of both wild-type and mutated crystallin proteins in a concentration-dependent manner, while cholesterol had no effect (Fig. 3e, f and Extended Data Fig. 5). We further showed that lanosterol, but not cholesterol, increased the amounts of mutant crystallins in the soluble fractions of cell lysates (Fig. 3g and Extended Data Fig. 6a). Using serial live-cell imaging of cells expressing a GFP-fused Y118D mutant of αA-crystallin, we showed that addition of lanosterol could effectively diminish crystallin aggregates with a half-life of 222 ± 8 min (Fig. 3h), whereas addition of DMSO or cholesterol did not reduce aggresome formation (Extended Data Fig. 6b). Single-particle tracking in live cells clearly showed that lanosterol has an important role in the dissociation of pre-formed intracellular protein aggregates.

To investigate whether lanosterol has a direct effect on the dissolution of aggregated proteins, the aggregates of five wild-type and nine mutant crystallins were obtained by heating wild-type and mutated crystallins in the presence of 1 M guanidine chloride. Under this condition, all crystallin proteins formed amyloid-like fibrils as revealed by the enhancement of thioflavin T (ThT) fluorescence, the fibrillar structures under negatively stained transmission electron microscopy (TEM), and the low turbidity value (Fig. 4 and Extended Data Fig. 6c).

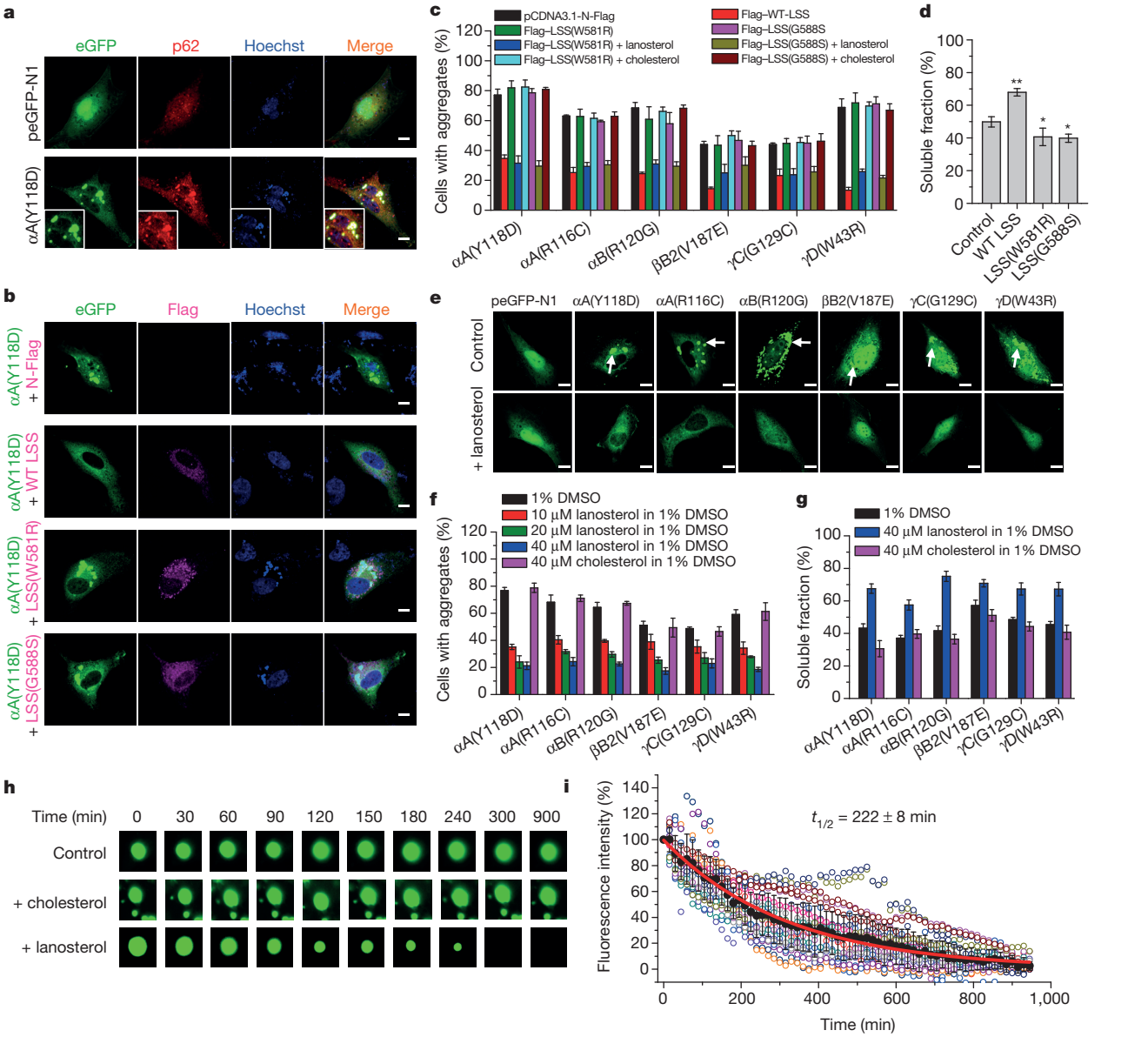

Figure 3 | Lanosterol reduced intracellular aggregation of various crystallin mutant proteins. **a**, Confocal images of crystallin protein aggregates in human lens progenitor cells. The cataract-causing Y118D mutant of αA-crystallin formed p62-positive intracellular inclusion bodies or aggresomes. Green, eGFP–crystallin proteins; red, p62; blue, nuclei. Cells transfected with peGFP-N1 were used as a control. **b**, Confocal images of the inhibitory effect of LSS on crystallin aggregates. **c**, Inhibition of crystallin mutant aggregation by wild-type LSS (WT LSS) and lanosterol, but not mutant LSS or cholesterol. **d**, Increase in soluble αA-crystallin(Y118D) mutant protein by co-expression of wild-type LSS but not LSS mutants (Y118D co-expressed with pcDNA3.1–N-Flag was used as a control). Quantitative analysis was performed using densitometry of crystallin proteins by western blot analysis of the supernatant or insoluble fraction of cell lysates. $n = 3$ in each group; representative western blot analysis is shown in Extended Data Fig. 4c; $^{*}P < 0.05$, $^{**}P < 0.01$. **e**, Confocal images of the re-dissolution of preformed crystallin aggregates by lanosterol. Arrows indicate the presence of crystallin aggregation. **f**, Lanosterol significantly reduced the intracellular aggregation by various cataract-causing mutant crystallin proteins in a concentration-dependent manner. $n = 3$; $P < 1 \times 10^{-4}$. Cholesterol did not reduce intracellular aggregation. $n = 3$; $P > 0.1$. **g**, Lanosterol increased the soluble fractions of various crystallin mutants in human lens progenitor cells. $n = 3$; $P < 0.001$. **h**, Effects of DMSO, cholesterol or lanosterol on αA-crystallin(Y118D) aggregates in human lens progenitor cells by serial live-cell imaging. Progression of crystallin aggregation dissolution by lanosterol can be observed, as evidenced by decreased green fluorescence following the time-course. **i**, Effect of lanosterol on dissolution of intracellular crystallin aggregates over time. $n = 22$ from three biological replicates. The 22 repetitions are shown in open circles distinguished by different colours. The mean ± s.d. values are shown as filled black circles and error bars. The data are best fitted by the single exponential decay process (red line). Scale bars, 10 μm.

The morphology of the amyloid-like fibrils obtained here was similar to those crystallin proteins reported previously[14]. We used PBS-containing liposomes formed by dipalmitoyl phosphatidylcholine (DPPC) to increase the solubility of sterol compounds and mimic the condition of sterols in cell membranes. Lanosterol, but not cholesterol, successfully re-dissolved the aggregated crystallin proteins from the amyloid-like fibrils in a concentration-dependent manner as indicated by the disappearance of fibrillar structures in the negatively stained TEM photographs and the decrease in ThT fluorescence intensity (Fig. 4 and Extended Data Fig. 6d). As an example, the re-dissolved αA-crystallins could be identified in negatively stained TEM pictures and were around 15 nm in size (Fig. 4a)[15].

To assess the effect of lanosterol on cataract reduction in lens tissues, we isolated naturally occurring cataractous lenses from rabbits. We incubated these cataractous lenses in a 25 mM lanosterol solution for 6 days and compared lens clarity before and after treatment of

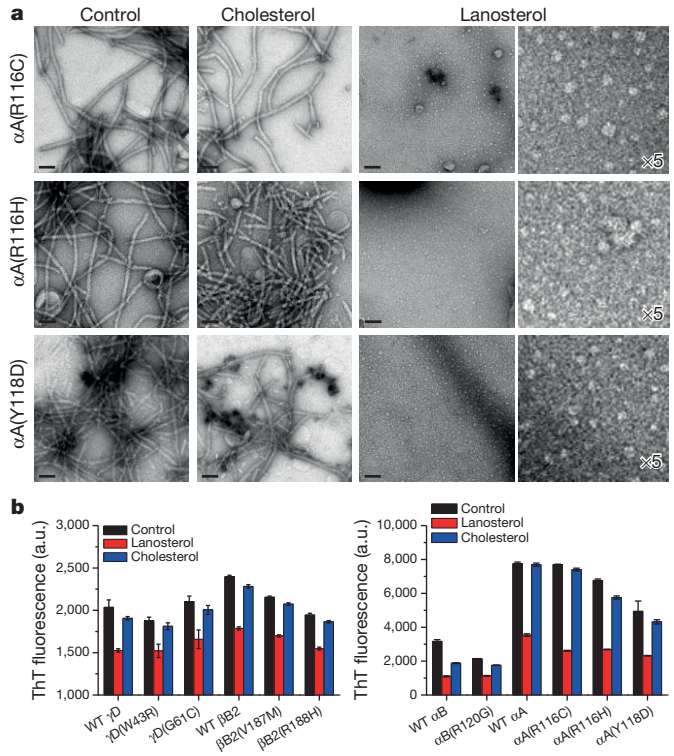

Figure 4 | Lanosterol re-dissolved pre-formed amyloid-like fibrils of crystallin proteins. **a**, Negatively stained TEM photographs of aggregates of αA-crystallin mutant proteins treated by a liposome vehicle, cholesterol or lanosterol in liposomes. Images in the right column of the lanosterol group show a 5× magnification of the image on their left. **b**, Effect of lanosterol on the re-dissolution of crystallin aggregates by ThT fluorescence ($n = 3$). Left, β/γ-crystallin mutants; right, α-crystallin mutants. Each bar results from three independent samples.

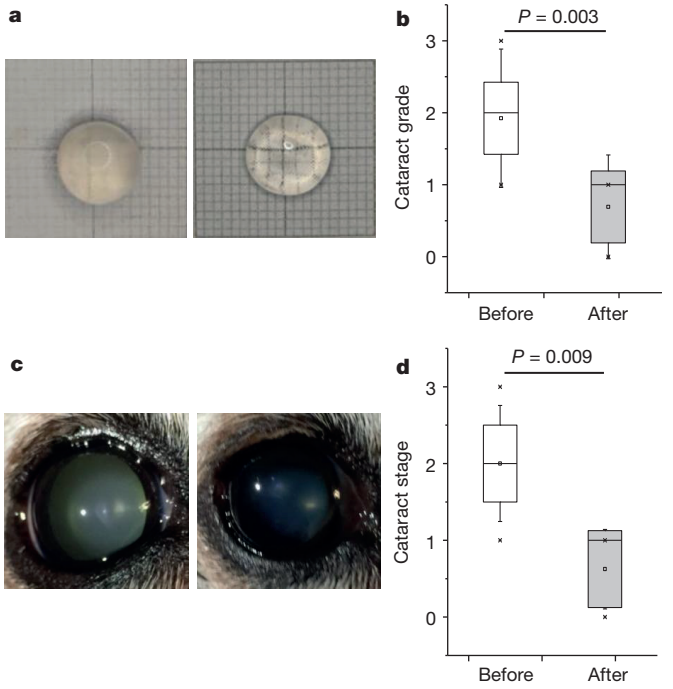

Figure 5 | Lanosterol reduced cataract severity and increased clarity. **a**, Photographs of a cataractous rabbit lens treated with lanosterol showing increased lens clarity. Left, before treatment; right, after. **b**, Boxplot of the quantification of the treatment effect of lanosterol ($n = 13$). **c**, Photographs of a cataractous dog lens treated with lanosterol showing increased lens clarity. Left, before treatment; right, after. **d**, Boxplot of the quantification of the treatment effect of lanosterol ($n = 7$). Range, median (horizontal line) and mean (circle) are presented. Crosses indicate the maximum and minimum cataract grades measured. Whiskers indicate the standard deviation and the box encompasses a 40% confidence interval.

lanosterol. We observed a strong trend of reduction in cataract severity, as demonstrated by an increase in lens clarity ($P < 0.003$, Wilcoxon Test, Fig. 5a, b, Extended Data Table 2a and Extended Data Fig. 7a, b). We further investigated the effect of lanosterol in reversing cataracts in dogs *in vivo*. Lanosterol treatment significantly reduced cataract severity and increased lens clarity ($P < 0.009$, Wilcoxon Test, Fig. 5c, d; Extended Data Table 2b and Extended Data Fig. 7c).

In this study, we demonstrated that homozygous mutations affecting the catalytic function of LSS cause extensive congenital cataracts with severe vision loss. The critical role of lanosterol in cataract prevention is supported by the observation that a rat strain harbouring compound *LSS* mutations recapitulates the human cataract disease phenotype[6]. Consistent with this notion, inhibition of LSS by U18666A, an LSS inhibitor (also known as an oxidosqualene cyclase inhibitor), was found to cause cataracts[16]. Furthermore, lanosterol treatment both decreased protein aggregation caused by mutant crystallin proteins in cell culture and reduced preformed cataract severity by increasing lens clarity in animal models. It is conceivable that the amphipathic nature of lanosterol allows it to intercalate into and coat hydrophobic core areas of large protein aggregates, effectively allowing these aggregations to gradually become water soluble again.

In summary, we show that lanosterol plays a key role in inhibiting lens protein aggregation and reducing cataract formation, suggesting a novel strategy for the prevention and treatment of cataracts. Cataracts are the leading cause of blindness and millions of patients every year undergo cataract surgery to remove the opacified lenses. The surgery, although very successful, is nonetheless associated with complications and morbidities. Therefore, pharmacological treatment to reverse cataracts could have large health and economic impacts. In addition, our results may have broader implications for the treatment of protein-aggregation diseases, including neurodegenerative diseases and diabetes, which collectively are a significant cause of morbidity and mortality in the elderly population, by encouraging the investigation of small-molecule approaches, such as the one demonstrated here.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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