Genetic and Functional Characterization of ANGPTL7 as a Therapeutic Target for Glaucoma

Kavita Praveen

Regeneron Genetics Center

Gaurang Patel

Regeneron <https://orcid.org/0000-0003-2497-665X>

Lauren Gurski

Regeneron Genetics Center

Ariane Ayer

Regeneron Genetics Center

Trikaladarshi Persaud

Regeneron Genetics Center

Matthew Still

Regeneron Genetics Center <https://orcid.org/0000-0002-3862-2425>

Lawrence Miloscio

Regeneron Pharmaceuticals

Tavé Van Zyl

Regeneron Pharmaceuticals

Silvio Alessandro Di Gioia

Regeneron Genetics Center

Ben Michael Brumpton

Norwegian University of Science and Technology <https://orcid.org/0000-0002-3058-1059>

Kristi Krebs

University of Tartu

Bjorn Olav Asvold

Norwegian University of Science and Technology <https://orcid.org/0000-0003-3837-2101>

Esteban Chen

Regeneron Genetics Center

Venkata Chavali

Scheie Eye Institute, University of Pennsylvania

Wen Fury

Regeneron Pharmaceuticals, Inc. <https://orcid.org/0000-0001-8671-8221>

Venkata Gudiseva

Scheie Eye Institute, University of Pennsylvania

Sarah Hyde

Alnylam Pharmaceuticals

Eric Jorgenson

Regeneron <https://orcid.org/0000-0002-5829-8191>

Stefanie Lefebvre

Alnylam Pharmaceuticals

Dadong Li

Regeneron Genetics Center

Alexander Li

Regeneron Genetics Center

James McIninch

Alnylam Pharmaceuticals

Brijeshkumar Patel

Regeneron Genetics Center

Jeremey Rabinowitz

Regeneron Pharmaceuticals

Rebecca Salowe

Scheie Eye Institute, University of Pennsylvania

Claudia Schurmann

Regeneron Genetics Center

Anne-Sofie Seidelin

Copenhagen University Hospital

Eli Stahl

Regeneron <https://orcid.org/0000-0002-1192-0561>

Dylan Sun

Regeneron Genetics Center

Tanya Teslovich

Regeneron Genetics Center

Anne Tybjærg-Hansen

Rigshospitalet

Cristen Willer

University of Michigan <https://orcid.org/0000-0001-5645-4966>

Scott Waldron

Alnylam Pharmaceuticals

Sabrina Walley

Regeneron Genetics Center

Hua Yang

Regeneron Pharmaceuticals

Sarthak Zaveri

Regeneron Pharmaceuticals

Ying Hu

Regeneron Pharmaceuticals

Kristian Hveem

Norwegian University of Science and Technology

Olle Melander

Lili Milani

University of Tartu <https://orcid.org/0000-0002-5323-3102>

Stefan Stender

Rigshospitalet <https://orcid.org/0000-0003-0281-5900>

Joan O'Brien

University of Pennsylvania

Marcus Jones

Human Longevity (United States)

Goncalo Abecasis

Regeneron Genetics Center, Tarrytown, NY

Michael Cantor

Regeneron Genetics Center

Jonathan Weyne

Regeneron Genetics Center

Katia Karalis

Regeneron

Aris Economides

Regeneron Pharmaceuticals <https://orcid.org/0000-0002-6508-8942>

Giusy Della Gatta

Regeneron Genetics Center

Manuel Ferreira

Regeneron Pharmaceuticals

George Yancopoulos

Regeneron Genetics Center, Regeneron Pharmaceuticals

Aris Baras

Regeneron Genetics Center <https://orcid.org/0000-0002-6830-3396>

Carmelo Romano

Regeneron

Giovanni Coppola (✉ giovanni.coppola@regeneron.com)

Regeneron Pharmaceuticals <https://orcid.org/0000-0003-2105-1061>

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Authors: Kavita Praveen¹, Gaurang C. Patel², Lauren Gurski¹, Ariane H. Ayer¹, Trikaladarshi Persaud¹, Matthew D. Still², Lawrence Miloscio¹, Tavé Van Zyl², Silvio Alessandro Di Gioia¹, Ben Brumpton^{3,4,5}, Kristi Krebs⁶, Bjørn Olav Åsvold^{3,4,7}, Esteban Chen¹, Venkata R. M. Chavali⁸, Wen Fury², Harini V. Gudiseva⁸, Sarah Hyde⁹, Eric Jorgenson¹, Stephanie Lefebvre⁹, Dadong Li¹, Alexander Li¹, James McIninch⁹, Brijeshkumar Patel², Jeremy S. Rabinowitz², Rebecca Salowe⁸, Claudia Schurmann^{1,10}, Anne-Sofie Seidelin¹¹, Eli Stahl¹, Dylan Sun¹, Tanya M. Teslovich¹, Anne Tybjærg-Hansen¹¹, Cristen Willer^{12,13,14}, Scott Waldron⁹, Sabrina Walley², Hua Yang², Sarthak Zaveri², Regeneron Genetics Center*, GHS-RGC DiscovEHR Collaboration*, Estonian Biobank Research Team*, Ying Hu², Kristian Hveem^{3,4}, Olle Melander^{15,16}, Lili Milani⁶, Stefan Stender¹¹, Joan M. O'Brien⁸, Marcus Jones¹, Gonçalo R. Abecasis¹, Michael N. Cantor¹, Jonathan Weyne², Katia Karalis¹, Aris Economides^{1,2}, Giusy Della Gatta¹, Manuel A. Ferreira¹, George D. Yancopoulos², Aris Baras¹, Carmelo Romano², Giovanni Coppola¹

Affiliations:

¹Regeneron Genetics Center, Tarrytown, NY 10591, USA.

²Regeneron Pharmaceuticals, Inc., Tarrytown, NY 10591, USA.

³K.G. Jebsen Center for Genetic Epidemiology, Department of Public Health and Nursing, NTNU, Norwegian University of Science and Technology, Trondheim, 7030, Norway.

⁴HUNT Research Center, Department of Public Health and Nursing, NTNU, Norwegian University of Science and Technology, Levanger, 7600, Norway.

⁵Clinic of Medicine, St. Olavs Hospital, Trondheim University Hospital, Trondheim, 7030, Norway.

⁶Estonian Genome Centre, Institute of Genomics, University of Tartu, Tartu, Estonia.

⁷Department of Endocrinology, Clinic of Medicine, St. Olavs Hospital, Trondheim University Hospital, Trondheim, 7030, Norway.

⁸Department of Ophthalmology, Pearlman School of Medicine, University of Pennsylvania.

⁹Alnylam Pharmaceuticals, Inc., Cambridge, MA 02142, USA.

¹⁰Bayer AG, Pharmaceuticals, Research and Development, 13342 Berlin, Germany

¹¹Department of Clinical Biochemistry, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark.

¹²Division of Cardiovascular Medicine, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA.

¹³Department of Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI, USA.

¹⁴Department of Human Genetics, University of Michigan, Ann Arbor, MI, USA.

¹⁵Lund University, Department of Clinical Sciences Malmö, Malmö, Sweden.

¹⁶Skåne University Hospital, Department of Emergency and Internal Medicine, Malmö Sweden.

*A complete list of contributing authors to the Regeneron Genetics Center, GHS-RGC DiscovEHR Collaboration and Estonian Biobank Research Team is provided in the Supplementary Appendix.

Correspondence to giovanni.coppola@regeneron.com, aris.baras@regeneron.com, or carl.romano@regeneron.com.

Abstract:

Glaucoma is a leading cause of blindness. Current glaucoma medications work by lowering intraocular pressure (IOP), a risk factor for glaucoma, but most treatments do not directly target the pathological changes leading to increased IOP, which can manifest as medication resistance as disease progresses. To identify physiological modulators of IOP, we performed genome- and exome-wide association analysis in >129,000 individuals with IOP measurements and extended these findings to an analysis of glaucoma risk. We report the identification and functional characterization of rare coding variants (including loss-of-function variants) in *ANGPTL7* associated with reduction in IOP and glaucoma protection. We validated the human genetics findings in mice by establishing that *Angptl7* knockout mice have lower (~2 mmHg) basal IOP compared to wild-type, with a trend towards lower IOP also in heterozygotes. Conversely, increasing m*Angptl7* levels via injection into mouse eyes increases the IOP. We also show that acute gene silencing via siRNA knockdown of *Angptl7* in adult mice lowers the IOP (~2-4 mmHg), reproducing the observations in knockout mice. Collectively, our data suggest that *ANGPTL7* is important for IOP homeostasis and is amenable to therapeutic modulation to help maintain a healthy IOP that can prevent onset or slow the progression of glaucoma.

Main text:

INTRODUCTION

Glaucoma is a leading cause of irreversible blindness, with a global prevalence of 3.54% in individuals 40-80 years of age, and is projected to affect more than 111.8 million people by 2040 (1). Classified as a neurodegenerative disease, glaucoma is characterized by the progressive loss of retinal ganglion cells in the eye and thinning of the neuroretinal rim of the optic nerve head.

Affected individuals present with visual field loss that is accompanied by increased intraocular pressure (IOP) in the majority of cases (2). Primary open angle glaucoma (POAG) is the most common glaucoma subtype and has highest prevalence in individuals of African ancestry (4.2% prevalence in Africa (1)).

Individuals at greatest risk for POAG are >60 years of age, have a family history of glaucoma, or have high myopia (3–6). Measurable ocular anatomical and physiological features, including low central corneal thickness (CCT), increased cup-to-disc ratio and high intraocular pressure (IOP) (7) correlate with increased risk for glaucoma and, like glaucoma (8), are highly heritable (4, 9–13). Thus, these quantitative risk factors, when measured on large numbers of individuals, can provide a well-powered dataset for genetic studies to elucidate the etiology of glaucoma risk and progression. The latest genome-wide association study (GWAS) of IOP included more than 130,000 individuals and increased the tally of IOP-associated loci to over 100 (14, 15). An earlier GWAS (16) reported that ~89% of loci associated with IOP at a genome-wide significant level showed directionally consistent effects on glaucoma risk, thus reinforcing the utility of quantitative risk factors in gene discovery for glaucoma.

Lowering IOP is the mainstay of all glaucoma therapeutics as IOP continues to be the only modifiable risk factor for the onset or progression of glaucoma. While many effective topical IOP-lowering agents across multiple drug classes are available, they suffer from significant drawbacks, including poor compliance due to frequent dosing requirements and side-effects (17). Waning efficacy over time and the consequent need for treatment escalation are also observed, perhaps in part because the majority of medications in use do not address pathophysiological changes at the

primary site of IOP regulation and aqueous humor egress from the eye, the trabecular meshwork (TM). These limitations result in a large proportion of glaucoma treatment regimens comprising more than one therapeutic agent and patients frequently changing medications or requiring treatment escalation that ultimately involves invasive surgery to maintain a clinically acceptable IOP (18). Therefore, a significant unmet need remains in the treatment of glaucoma to identify new therapeutic targets offering novel mechanisms of action, as well as treatment platforms that may offer increased durability and tolerability without compromising safety.

We performed genetic association analyses of IOP and glaucoma across eight cohorts to identify rare and coding variants that modulate the risk for glaucoma through IOP. This led to the identification of *ANGPTL7* as a candidate, consistent with findings reported recently by another group (19). In this report, we 1) strengthen the genetic link to glaucoma protection by (i) showing a consistent protective effect of the Gln175His variant in *ANGPTL7* across eight cohorts, (ii) identifying a burden of ultra-rare missense variants in *ANGPTL7* that reduce IOP levels, and (iii) identifying an additional rare *ANGPTL7* loss-of-function variant in African-ancestry individuals; we also present 2) *in vitro* characterization of *ANGPTL7* variants identified from genetic analyses; and 3) *in vivo* results showing that mice without *Angptl7* have reduced basal IOP, and that even a partial knockdown of *Angptl7* with siRNA can lead to lowering of IOP in mice. Our results establish an important role for *ANGPTL7* as a physiological regulator of IOP and suggest that it is also amenable to modification by pharmacological tools, making it a compelling target for a glaucoma therapeutic.

RESULTS

Coding variants in *ANGPTL7* are associated with reduced IOP

We studied the effect of rare, protein-altering variation on IOP across two large cohorts, UK Biobank (UKB) and Geisinger DiscovEHR (GHS), including 129,207 individuals of European descent after exclusion of cases with a glaucoma diagnosis (Methods, Table S1). To increase our power to detect associations with rare variants, we performed burden tests by aggregating for each gene all rare (minor allele frequency (MAF) < 1%) predicted loss-of-function (pLOF, defined as stop-gain, frameshift, splice donor, splice acceptor, start-loss, and stop-loss) and missense (predicted deleterious by 5 algorithms, Supplementary Methods) variants. We observed a genome-wide significant association ($P < 5 \times 10^{-8}$) of variants in angiopoietin-like 7 (*ANGPTL7*) with reduced IOP ($\beta_{\text{allelic}} = -0.21$, $P = 5.3 \times 10^{-24}$; Figure 1A). The gene burden included 63 rare variants but was dominated by two: a missense (Gln175His, MAF = 0.7%) and a stop-gain (Arg177*, MAF = 0.03%) variant, which accounted for 1,902 and 82 individuals out of a total of 2,188 carriers, respectively (Figure 1B, 2, S1). Exclusion of Gln175His and Arg177* from the burden meta-analysis between UKB and GHS did not eliminate the signal completely ($\beta_{\text{allelic}} = -0.23$, $P = 4.4 \times 10^{-4}$; Figure S2), suggesting that other ultra-rare variants in *ANGPTL7* are also associated with reduced IOP.

In single-variant analyses, Gln175His was associated with reduced IOP at a genome-wide significant level ($\beta_{\text{allelic}} = -0.20$ SD, $P = 3.1 \times 10^{-20}$, Figure 2A). Heterozygous and homozygous carriers of Gln175His in *ANGPTL7* have a 5.2% (0.8 mmHg) and 26.5% (4.1 mmHg) reduction in median IOP in UKB, respectively (Figure 2B). The Arg177* variant was also nominally associated with reduced IOP with an effect size similar to that of Gln175His ($\beta_{\text{allelic}} = -0.24$ SD, $P = 2.6 \times$

10⁻², Figure 2C), and 77 heterozygous Arg177* carriers had a 9% (1.4 mmHg) median IOP decrease (Figure 2D). Arg177* appears to be the predominant pLOF variant in European populations; a burden test restricted to pLOF variants (15 variants across UKB and GHS) was dominated by Arg177* (82 of 112 total carriers) and was comparable ($\beta_{\text{allelic}} = -0.21$ SD, $P = 2.2 \times 10^{-2}$; Figure S3) to the single-variant association of Arg177* with IOP.

We searched other ancestries for additional pLOFs in *ANGPTL7* and identified Trp188*, which is enriched in individuals of African descent (MAF = 0.3%) compared to Europeans (MAF = 0.0013%). We performed an association of Trp188* with IOP in African ancestry individuals from UKB and the Primary Open Angle African American Glaucoma Genetics (POAAGG) study, followed by meta-analysis. Trp188* showed a trend towards reduced IOP, similar to Arg177* and Gln175His, but this was not statistically significant ($\beta_{\text{allelic}} = -0.11$ SD, $P = 5 \times 10^{-1}$). A cross-ancestry meta-analysis of Arg177* and Trp188* variants showed a nominally significant association with reduced IOP ($\beta_{\text{allelic}} = -0.21$ SD, $P = 1.5 \times 10^{-2}$; Figure S4).

In summary, we observed a significant association of Gln175His in *ANGPTL7* with reduced IOP and a sub-threshold association, in the same direction and of similar magnitude, with pLOF variants in *ANGPTL7*. Assuming that the pLOF variants indeed cause a loss of protein function, our data suggests that loss of *ANGPTL7* can lead to lower IOP.

IOP-associated variants in *ANGPTL7* are protective against glaucoma

To understand if carriers of variants in *ANGPTL7* would also be protected against glaucoma, we performed an association analysis of Gln175His with glaucoma in UKB, GHS, and

six additional studies: Mount Sinai's BioMe Personalized Medicine Cohort from Mount Sinai Health System, New York (SINAI), the Malmö Diet and Cancer Study from Malmö, Sweden (MALMO), the FinnGen cohort from Finland, the Estonia Biobank at the University of Tartu, Estonia (EstBB), the HUNT study from Nord-Trøndelag, Norway (HUNT), and the Copenhagen General Population Study/Copenhagen City Heart Study from Copenhagen, Denmark (CGPS-CCHS). A meta-analysis across these eight cohorts showed a significant reduction in glaucoma risk for Gln175His carriers (odds ratio (OR_{allelic}) = 0.77, $P = 2.7 \times 10^{-6}$, Figure 3A). We also analyzed glaucoma risk in carriers of the rarer Arg177*/Trp188* variants in a cross-ancestry meta-analysis and observed a consistent trend towards reduction in risk ($OR_{\text{allelic}} = 0.87$, $P = 4.1 \times 10^{-1}$, Figure 3B). Taken together, the associations of missense and pLOF variants in *ANGPTL7* with reduced IOP and the association of the missense variant with reduced glaucoma risk suggest the hypothesis that loss of *ANGPTL7* confers protection against glaucoma, and that this effect is mediated through the regulation of IOP.

***ANGPTL7* variants are associated with corneal measures**

We performed a phenome-wide association analysis (PheWAS) to understand whether other traits were associated with a burden of pLOF and deleterious missense variants in *ANGPTL7*. We tested the *ANGPTL7* variant aggregate for association with 14,050 and 10,032 binary and quantitative traits in UKB and GHS, respectively. No associations reached phenome-wide significance ($P < 2 \times 10^{-6}$ after multiple-testing correction for 24,082 total traits) in GHS. The only significant associations in UKB were with ocular traits (Table 1), specifically with decreased IOPcc, decreased corneal resistance factor (CRF) and increased corneal refractive power along both weak and strong meridians measured at 3 and 6mm diameters. The effect of these variants on

IOPcc was slightly attenuated (-0.17 SD) compared to that on IOPg (-0.22 SD in UKB), which suggests that *ANGPTL7* has some impact on corneal properties that are known to affect the IOPg measurements (20). The association observed with decreased CRF is also consistent with a corneal effect of *ANGPTL7*.

We used the autorefraction measurements at 3mm diameter to derive measures of clinical interest, namely, mean corneal refractive power (mCRP), corneal astigmatism, and refractive astigmatism (Supplementary Methods) and checked for association with *ANGPTL7*. We observed a significant association with increased mCRP ($\beta_{\text{allelic}} = 0.16$, $P = 1.1 \times 10^{-13}$, Table 1) but no association with corneal or refractive astigmatism. We also did not observe associations with mean spherical equivalent (MSE; measure of refractive error) or myopia (either derived from MSE or via ICD-10 diagnosis), which could result from increased mCRP (Table S2). Overall, our PheWAS results show that while *ANGPTL7* is associated with changes in corneal anatomy/biomechanics-related quantitative measures, we did not detect an increased risk for any related disease outcomes that we could test. In addition, pLOF and deleterious missense variants in *ANGPTL7* are not associated with any systemic quantitative traits or binary outcomes.

Gln175His, Arg177* and Trp188* are defective in secretion

To understand the impact of Gln175His, Arg177*, and Trp188* variants on the expression and secretion of *ANGPTL7*, we transiently transfected constructs expressing the wild type (WT), Gln175His, Arg177*, and Trp188* variant proteins in HEK293 cells. We measured mRNA levels by Taqman, which showed similar Gln175His and Arg177* transcript levels and a trend towards decreased levels of the Trp188* transcript compared to WT ($P = 0.08$; Figure S5). However,

analysis of intracellular, steady-state protein in whole-cell lysate by western blotting and ELISA revealed increased levels of Gln175His compared to WT ($P < 1 \times 10^{-4}$; Figure 4C). As expected, Arg177* and Trp188* encoded lower molecular weight proteins (~30-32 kDa). No significant difference in the protein levels of these two mutants was revealed by ELISA in comparison to WT (Figure 4A, C). Because ANGPTL7 is a secreted protein, we next determined the levels of WT, Gln175His, Arg177*, and Trp188* in the cellular supernatant. Protein analysis by Western blot showed that the Arg177* and Trp188* variants were not detectable and the Gln175His was drastically reduced in the supernatant compared to WT ($P < 0.05$; Figure 4B). ELISA assay further corroborated the severely reduced levels of Gln175His and the inability of Arg177* and Trp188* to reach the extracellular space (Figure 4C, D).

ANGPTL7 is expressed in cornea, TM and sclera across species

To identify expression of *ANGPTL7* in ocular tissues across different species, transcriptome profiles from different parts of eye were generated (Supplementary Methods). High *ANGPTL7* expression was observed in cornea, TM, and sclera in human and African green monkey eyes (Figure 5A, B). High *Angptl7* expression was also observed in cornea, TM, sclera, optic nerve, and choroid/RPE in eyes of C57BL/6J mice (Figure 5C). *In situ* hybridization on human donor and mouse eyes using RNAscope probes for human *ANGPTL7* and mouse *Angptl7* showed *ANGPTL7/Angptl7* expression in TM, cornea stroma, and sclera (Figure 5D, E; Supplementary Methods).

Increasing levels of *Angptl7* in mouse eyes increases IOP

Previous studies showed that overexpression of *ANGPTL7* in TM cells leads to changes in extracellular matrix (ECM) deposition and reorganization (21, 22) and that *ANGPTL7* is increased in aqueous humor of glaucoma patients (22), however, the role of *ANGPTL7* in IOP regulation is not clear. To investigate this, we injected *Angptl7* protein in mice via intravitreal and intracameral routes and measured IOP over time. Intravitreal injection of *Angptl7* protein in mice led to an initial drop in IOP followed by, starting on day 4, an elevation in IOP of 4-5 mmHg, a 22-25% increase compared to baseline, that lasted until the end of the experiment on day 7 (Figure 6A). Similarly, intracameral injection of *Angptl7* protein in mice led to an initial drop and subsequent elevation (by 2-5 mmHg) of IOP, starting on day 3 until the end of the experiment on day 7 (Figure 6B). Vehicle-injected mice did not show an increase in IOP in either route of administration.

***Angptl7* KO mice have lower basal IOP than WT**

We generated and characterized *Angptl7*^{-/-} (KO) mice. We did not observe any ocular changes on anterior segment optical coherence tomography (OCT), or a difference in corneal thickness between the two genotypes (Figure S6). We also monitored the IOP in KO, *Angptl7*^{+/-} (Het) and WT mice and observed a dose-dependent decrease in IOP across the three genotypes (Figure 7A). The mean IOP was lowered in KO mice (mean $\pm$ standard error of the mean (SEM): 15.39 ± 0.25 mmHg) by 11% (1.96 mmHg, $P < 1 \times 10^{-4}$) compared to WT (17.36 ± 0.23 mmHg). Het mice (16.26 ± 0.43 mmHg) showed a smaller (6%, 1.1 mmHg, $P = 0.02$) but significant reduction in IOP compared to WT. We confirmed via RNAscope that *Angptl7* mRNA was not expressed in any ocular tissue in KO mice whereas it was expressed in TM, cornea, and sclera of WT mice (Figure S7; Supplementary Methods).

siRNA-induced knockdown of Angptl7 mRNA and lowering of IOP in WT Mice

To investigate whether knockdown of Angptl7 with small interfering RNA (siRNA) can also lower IOP, we tested six different siRNAs targeting Angptl7 in C57BL/6J mice and monitored IOP over time. We injected C57BL/6J mice intravitreally with 15 μ g of siRNAs and performed qPCR six weeks later on limbal rings dissected from mouse eyes enriched for the TM. IOP was significantly lowered 2 weeks post-injection in mice treated with two of the six siRNAs (siRNA #3 and #5) compared to the PBS and Naïve (no injection) groups (Figure 7B). Naïve and PBS-treated animals maintained their IOPs at baseline for the duration of the study (weeks 0-6). In mice treated with siRNA#3 and #5, IOP was lowered by 2-4 mmHg starting at week 2 compared to PBS-treated mice (Figure 7B). At the end of the study, we collected the eyes, carefully micro-dissected the limbal ring and performed qPCR. We observed the highest level of knockdown (>50%) of Angptl7 mRNA with siRNAs #3 and #5 compared to PBS-treated mice, which is consistent with the IOP lowering observed in mice injected with these two siRNAs (Figure 7C). These results suggest that acute inhibition of Angptl7 expression also lowers IOP.

DISCUSSION

In this study, we present genetic and functional evidence for a role for *ANGPTL7* in the physiological control of IOP and as a potential target for glaucoma therapy. Through genetic association analyses across 8 cohorts in Europeans, we confirmed the association of a rare missense variant, Gln175His (rs28991009), in *ANGPTL7* with a decrease in IOP and with decreased risk for glaucoma (15, 19). We further identified pLOF variants in *ANGPTL7*, Arg177* (rs143435072) and African ancestry-enriched Trp188* (rs145750805), that also associated with a decrease in IOP, suggesting that Gln175His carriers are protected from glaucoma through a loss or reduction in *ANGPTL7* activity. Through cell-based expression assays, we found that Gln175His, Arg177*, and Trp188* were severely defective in secretion when compared to wild-type and, while not proof, this observation is consistent with the hypothesis that they result in a loss of protein function. We also identified predicted-deleterious *ANGPTL7* variants in burden analyses associated with reduced IOP, indicating that there may be other ultra-rare *ANGPTL7* variants that confer protection from glaucoma. Supporting this hypothesis, a recent report described the association of one of these *ANGPTL7* variants enriched in Finnish individuals (Arg220Cys) with reduced IOP and decreased risk for glaucoma (19).

ANGPTL7 is a secreted glycoprotein that is thought to form homo-oligomers (tri- or tetramers), like other members of the *ANGPTL* family (23, 24). The *ANGPTL7* mRNA was first isolated from human postmortem eyes where it shows the highest expression relative to other tissues (23). Within the eye, our in situ hybridization results show that *ANGPTL7* is expressed most strongly in the cornea and TM, consistent with previous findings (22). We have also shown using single-cell RNAseq that *ANGPTL7* expression is particularly enriched in the

juxtacanalicular tissue (JCT), a region of the TM most important for IOP regulation and generation of aqueous humor outflow resistance (25, 26). In addition to being expressed in tissues directly relevant in glaucoma, several lines of evidence have implicated *ANGPTL7* in glaucoma pathophysiology. First, elevated levels of *ANGPTL7* mRNA and protein were observed in eye tissues from glaucoma patients compared to controls, and under conditions of increased IOP simulated by perfusion of eye anterior segment explants (22, 27, 28). Second, *ANGPTL7* is one of the most upregulated genes in response to corticosteroid treatment (29, 30), which can cause increased IOP in ~40% of general population and ~90% of individuals with POAG (31, 32). Third, *ANGPTL7* levels are also increased in response to TGF-beta, a growth factor that is thought to modulate the ECM and lead to increased IOP (33). Fourth, *ANGPTL7* itself can modulate the expression of components of the TM ECM (21, 22).

The above studies suggest a strong correlation of *ANGPTL7* expression with glaucoma disease-state, however, they are not evidence for a causal relationship between *ANGPTL7* levels and elevated IOP/glaucoma. Human genetics finding of an association between *ANGPTL7* loss and lower IOP suggests that *ANGPTL7* is physiologically important for IOP regulation. We validated this hypothesis in mice where we performed reciprocal experiments: measuring IOP after increasing *ANGPTL7* levels via injection of mAngptl7 into mouse eyes, and after removing all mAngptl7 protein by generating *Angtpl7* KO mice. Our findings show that increasing mAngptl7 results in increased (~2-4 mmHg) IOP and decreasing mAngptl7 through KO mice reduces basal IOP levels (~2 mmHg), establishing that *ANGPTL7* functions *in vivo* to maintain IOP homeostasis. We further recapitulated the reduction in IOP observed in KO mice by injecting WT mouse eyes with siRNA against mAngptl7, which not only replicates the observation in genetic

mutant mice but also illustrates that the effect of mAngptl7 on IOP continues post-development and is amenable to modulation by therapeutics in adulthood. Our results of lower IOP in *Angptl7* KO mice are highly consistent with observations from human genetics. Based on this, we could extend the siRNA knockdown findings to humans and surmise that inhibition of ANGPTL7 in adulthood could be an efficacious way to lower IOP and, eventually, the risk for glaucoma.

Our human genetics findings indicate that the variants in *ANGPTL7* associated with reduced IOP were also associated with decreased mean corneal resistance factor (CRF), an output of the Ocular Response Analyzer (ORA) that reflects the elastic property of the cornea (34), and increased mean corneal refractive power (mCRP). mCRP, along with axial length and lens power, contributes to the overall refractive state of the eye (35), and increased mCRP (i.e. a steeper cornea) may be indicative of possible myopia or astigmatism. However, as we did not observe evidence of association with myopia or astigmatism in PheWAS, we conclude that the changes to mCRP are either insufficient to result in a disease outcome or are compensated by other changes in traits that were not measured, such as axial length (36). While an effect of ANGPTL7 on corneal properties is clearly indicated by the associations with mCRP and CRF, we assert that this effect cannot account for the full extent of IOP reduction observed. Instead, we posit an independent contribution of ANGPTL7 to IOP homeostasis based on the following: (1) The persistent association with reduced IOP_{cc} suggests that there is IOP reduction even after controlling for corneal properties; (2) *Angptl7* KO mice eyes show reduced basal IOP without evidence of corneal thinning or other corneal abnormalities; and (3) the association of ANGPTL7 variants with glaucoma protection is a result we would not expect if the reduction of IOP was purely due to

ANGPTL7's effect on corneal anatomy (37). Therefore, we believe that ANGPTL7 likely has a pleiotropic effect on both IOP and corneal anatomy/biomechanics in humans.

Therapeutic knockdown of ANGPTL7 in patients with open angle glaucoma may present a unique opportunity to not only lower IOP through a novel mechanism of action, but also to intervene in the disease process. Among the most widely used IOP-lowering agents, none directly address pathophysiological changes occurring at the level of TM and instead reduce IOP through mechanisms that reduce the aqueous burden on the conventional pathway, either through suppressing aqueous formation or increasing its outflow via the alternative (uveoscleral) pathway (38). Consequently, as TM dysfunction progresses in a glaucoma patient, treatment intensification naturally follows (18). While further studies are needed to investigate the role of ANGPTL7 in pathways contributing to TM dysfunction and IOP elevation, several lines of evidence point to pro-fibrotic actions at the level of the TM, including its corticosteroid and TGF-beta responsiveness, as well as its enriched expression in JCT TM (discussed earlier).

As all studies, this study has limitations. First, we did not account for IOP-lowering medication use in the IOP analysis. We did exclude from the IOP analysis individuals with a glaucoma diagnosis, greatly reducing the effect of medication use, however this would not exclude those individuals on IOP-lowering medications without a glaucoma diagnosis. In addition, the IOP measurement in UKB for most participants was only taken once, and therefore can be prone to errors that a median of multiple measurements might buffer against. Both of the above factors would influence the estimation of the effect of rare genetic variants on IOP. Second, since a glaucoma diagnosis can often be made well after the onset of disease, we can expect that a certain

percentage of controls have undiagnosed glaucoma, which may affect the effect size estimate. Third, the *ANGPTL7* gene in humans lies within intron 28 of *MTOR* (39), therefore variants in *ANGPTL7* could also affect MTOR function. While a possibility, it is unlikely that the effect on IOP and glaucoma is due to MTOR, as: (i) the variants are predicted to be protein-altering in *ANGPTL7* whereas there is no obvious predicted functional effect on MTOR; (ii) we show data suggesting that the variants have a functional impact on *ANGPTL7*; and (iii) we show mouse data that establish a role for *ANGPTL7* in IOP regulation.

In summary, our genetic and pharmacological results indicate that *ANGPTL7* participates in the normal physiological regulation of IOP in humans and mice. Since excessive amounts of *ANGPTL7* protein in the eyes of experimental animals cause IOP to elevate to pathological levels, upregulation of *ANGPTL7* in humans may be responsible for the elevated IOP that leads to POAG. Therefore, we propose *ANGPTL7* as an excellent candidate to explore as a therapeutic target for POAG.

MATERIALS AND METHODS

Study Design

Association with IOP was tested on a total of 101,678 individuals and 27,529 individuals of European ancestry from the United Kingdom Biobank (UKB) and the MyCode Community Health Initiative cohort from Geisinger Health System (GHS), respectively. The UKB is a population-based cohort study of people aged between 40 and 69 years recruited through 22 testing centers in the UK between 2006 and 2010 (40). The GHS MyCode study is a health system-based cohort of patients from Central and Eastern Pennsylvania (USA) recruited in 2007-2019 (41). For

IOP association tests in African ancestry individuals, we included 4,114 individuals from UKB and 3,167 individuals from the Primary Open Angle African-American Glaucoma Genetics (POAAGG) study conducted at the University of Pennsylvania Perelman School of Medicine (42). We excluded all participants with a glaucoma diagnosis code (ICD-10 H40) or self-reported glaucoma (UKB field IDs: 6148 and 20002) from IOP analyses.

Association with glaucoma was tested in 8 studies: UKB, GHS, Mt. Sinai BioMe cohort (SINAI), the Malmö Diet and Cancer study (MALMO) (43), the Estonia Biobank (EstBB) (44), The Trøndelag Heath Study (HUNT) (45), FinnGen, a study from Finland, and the Copenhagen General Population Study and the Copenhagen City Heart Study (CGPS-CCHS) (46). We had, in total, up to 40,042 cases (UKB: 12,377, GHS: 8,032, SINAI: 409, MALMO: 2,395, EstBB: 7,629, HUNT: 3,874; CPGS-CCHS: 1,863; FinnGen: 3,463) and 947,782 controls of European ancestry, and 5,153 cases (UKB: 448, POAAGG: 3,444, SINAI: 1,261) and 21,650 controls of African ancestry in glaucoma analyses.

Phenotype definition

IOP in UKB was measured in each eye using the Ocular Response Analyzer (ORA, Reichert Corp., Buffalo, New York). Participants were excluded from this test if they reported having eye surgery in the preceding 4 weeks or having an eye infection. The ORA calculates two forms of IOP, a Goldmann-correlated IOP (IOPg) and a corneal compensated IOP (IOPcc). IOPg most closely approximates the IOP measured by the Goldmann Applanation Tonometer (GAT), which has been the gold standard for measuring IOP, while IOPcc provides a measure of IOP that is adjusted to remove the influence of corneal biomechanics (47). For this study, we focused on

IOPg as this measurement is the most comparable to IOP measurements in other cohorts, and herein IOPg will be referred to as IOP. IOP in POAAGG was measured using a GAT. In GHS, IOP measurements were obtained from several instruments including GAT, Tono-pen and I-Care, which are correlated with IOPg readings from the ORA (48). For GHS individuals who were not prescribed any IOP medications, we used the median of all IOP measurements available. For individuals who had an IOP medication prescribed, we used the median of IOP measurements available preceding the start date for IOP medications (if available). Individuals for whom we did not have non-medicated IOP values were excluded from the IOP genetic analyses. For association analyses of IOP, we excluded individuals with: 1) a glaucoma diagnosis; 2) IOP measures that were more than 5 standard deviations away from the mean; 3) more than a 10-mmHg difference between both eyes. We derived a mean IOP measure between both eyes for each individual. IOP of only one eye was used in instances where IOP measures for both eyes were not available.

Details on glaucoma definition in each cohort are given in the Supplementary Methods. In brief, glaucoma cases in GHS, SINAI, MALMO, HUNT, EstBB, FinnGen and CGPS-CCHS were defined by the presence of an ICD-10 H40 diagnosis code in either outpatient or inpatient electronic health records. In UKB, glaucoma cases were defined as individuals with either an ICD-10 H40 diagnosis or self-reported glaucoma (UKB field ID: 6148 or 20002). In the POAAGG cohort, glaucoma cases and controls were classified based on an ophthalmic examination by glaucoma specialists, and glaucoma suspects were also included in the cases (49).

Statistical analyses

High coverage whole exome sequencing and genotyping was performed at the Regeneron Genetics Center as previously described (50, 51) and given in Supplementary Methods. We estimated the association with IOP and glaucoma of genetic variants or their gene burden using REGENIE v1.0.43 (52) (UKB, GHS, MALMO, SINAI), SAIGE (53) (HUNT, EstBB, FinnGen) or logistic regression (CGPS-CCHS). Analyses were adjusted for age, age², sex, an age-by-sex interaction term, experimental batch-related covariates, and genetic principal components, where appropriate. Cohort-specific statistical analysis details are provided in Supplementary Methods. Results across cohorts were pooled using inverse-variance weighted meta-analysis. Details on the PheWAS analysis conducted in UKB and GHS are provided in Supplementary Methods. Description of statistical analyses for in vitro and in vivo experiments are provided in the respective figure legends.

Functional studies

In vitro characterization was conducted for the WT ANGPTL7 and three ANGPTL7 variants: Gln175His, Arg177* and Trp188*. Briefly, WT ANGPTL7 and the three variants in a pcDNA 3.1(+) vector backbone were transiently transfected with FuGENE6 (Promega) into HEK293 cells. Protein and mRNA levels of the transfectants in the cell lysate and supernatant were measured via ELISA and TaqMan, respectively, and visualized by western blotting. Details are provided in Supplementary Methods.

Angptl7^{-/-} mice were generated by Regeneron Pharmaceuticals using the VelociMouse® technology (54). Heterozygous mice (*Angptl7*^{+/-}) were bred to generate age-matched wild-type, het and KO littermates that were used for experimentation. Ocular anatomy in these mice was characterized using optical coherence tomography (OCT). Detailed methods on generation and

444 characterization of KO mice, and injection of mAngptl7 and siRNA into mouse eyes are given in
445 Supplementary Methods.

446 IOP was measured as previously described (55–57). Briefly, mice were anesthetized and
447 IOP was measured in both eyes using a TonoLab rebound tonometer (Colonial Medical Supply,
448 Franconia, NH) before the start of Angptl7 injection and every day afterwards for six days. When
449 testing *Angptl7* siRNAs, IOPs were measured in each eye before then start of experiment and then
450 every week until end of study. IOP measurements for both eyes were completed within 3–5
451 minutes.

452

453 **Supplementary Materials**

454 Supplementary Appendix

455 Supplementary Methods

456 Table S1: Number of samples across cohorts included in IOP and glaucoma analyses.

457 Fig. S1: Missense and predicted loss-of-function (pLOF) variants in *ANGPTL7* and IOP levels in
458 individuals of European descent in GHS.

459 Table S1: Number of samples across cohorts included in IOP and glaucoma analyses.

460 Fig. S2: Meta-analysis of *ANGPTL7* aggregate of predicted loss-of-function and deleterious
461 missense variants (MAF<1%), excluding Gln175His and Arg177*, with IOP.

462 Fig. S3: Meta-analysis of *ANGPTL7* aggregate of predicted loss-of-function variants only
463 (MAF<1%) with IOP.

464 Fig. S4: Cross-ancestry meta-analysis of Arg177* and Trp188* with IOP.

465 Table S2: Association of *ANGPTL7* aggregate of pLOF and deleterious missense variants and
466 ocular traits of interest in UKB.

467 Fig. S5: Relative expression of WT and variant *ANGPTL7* mRNA in a HEK293 cell line.

468 Fig. S6: Characterization of *Angptl7* KO and WT mouse eyes by OCT.

469 Fig. S7: *In situ* characterization of *Angptl7* mRNA in WT and *Angptl7* KO mouse eyes.

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798 Conceptualization: KP, GP, AB, CR and GC

799 Data curation: DL, MNC

800 Genetic Analysis: KP, LG, MAF, AHA, SADG, AL, TMT, CS, DS, KK, BB, SS, ES, EJ

801 Funding acquisition: GDY, GRA, AB, JOB, CR, LM, OM, SS, KH

802 Mouse model and experiments: GP, BP, SWalley, HY, WF, SZ, JR, WF

803 siRNA design and development: SL, JM, SWaldron, SH

804 In vitro experiments: GDG, TP, LM, MDS

805 Project administration: EC, MJ

806 Datasets: JOB, VRMC, HVG, RS, BB, KH, BOA, CW, KK, LM, SS, AS, AT, OM

807 Supervision: MAF, GDG, YH, KH, LM, KK, AE, JW, AB

808 Writing – original draft: KP, GP, GDG, TVZ, CR, GC

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Figures

A

B

Figure 1: An aggregate of rare (MAF < 1%) loss-of-function and missense variants in *ANGPTL7* is associated with IOP. (A) Association of an aggregate of 63 pLOF and deleterious (based on 5 prediction algorithms) missense variants in *ANGPTL7* with reduced IOP in 129,207 individuals of European descent. (B) Missense and predicted loss-of-function (pLOF) variants in *ANGPTL7* and IOP levels in individuals of European descent from UKB. The plots represent Goldmann-correlated IOP (mean of both eyes) levels in carriers of 1 pLOF and 10 missense variants in *ANGPTL7* that are predicted deleterious by five different algorithms and have at least five carriers amongst the 101,678 exome-sequenced individuals with IOP measurements in the UK Biobank. The median IOP level across carriers of all 49 pLOF and predicted-deleterious missense *ANGPTL7* variants (14.64 mmHg) is indicated by the red line, and the median IOP in non-variant carriers (15.46 mmHg) is indicated by the blue line. Magenta diamonds mark the median IOP in carriers of each variant. Beneath the plots is the median and interquartile range of IOP and the numbers of variant carriers diagnosed with glaucoma or controls in UKB (n= 385,040).

Figure 2: Gln175His and Arg177* are major contributors to the gene burden association of *ANGPTL7* with IOP. Association of Gln175His (A) and Arg177* (C) variants in *ANGPTL7* with IOP, effect measured in standard deviation units, in individuals of European descent. (B, D) Boxplots representing Goldmann-correlated (IOPg) in the UK Biobank across genotypes. (B) Gln175His heterozygous and homozygous carriers have a 0.8-mmHg and 4.1-mmHg lower median IOPg, respectively, compared to non-carriers. (D) Arg177* heterozygous carriers have a

1.4-mmHg lower IOPg compared to non-carriers. GHS: Geisinger DiscovEHR, UKB: UK Biobank.

A

B

Figure 3: Association of *ANGPTL7* variants with glaucoma. (A) Meta-analysis results for Gln175His with glaucoma across 8 different cohorts. **(B)** Cross-ancestry meta-analysis of Arg177* and Trp188* across 5 EUR and 3 AFR cohorts. The variants in the meta-analysis of EUR and AFR cohorts were Arg177* and Trp188*, respectively. GHS: Geisinger DiscovEHR, UKB: UK Biobank, SINAI: Mt. Sinai Medical School BioMe Biobank, MALMO: Malmo Diet and Cancer

Study, EstBB: the Estonia Biobank at the University of Tartu; HUNT: the HUNT study from Nord-Trøndelag; CGPS-CCHS: the Copenhagen General Population Study and the Copenhagen City Heart Study, POAAGG: Primary Open Angle African-American Glaucoma Genetics, AAF = alternative allele frequency.

Table 1: Statistically significant ($P < 2 \times 10^{-6}$) results from PheWAS of an aggregate of up to 110 pLOF and deleterious missense variants (MAF<1%) in *ANGPTL7* in UKB. CRF=corneal resistance factor.

Trait	P-value	OR/Effect in SD (LCI UCI)
Intraocular pressure Corneal Compensated (IOPcc) (mean of both eyes)	6.32E-14	-0.17 [-0.21 -0.13]
CRF (right eye)	5.20E-13	-0.16 [-0.20 -0.11]
CRF (left eye)	4.50E-12	-0.15 [-0.20 -0.11]
6mm weak meridian (left eye)	4.00E-20	0.21 [0.16 0.25]
6mm weak meridian (right eye)	4.20E-18	0.19 [0.15 0.24]
6mm strong meridian (left eye)	3.10E-17	0.19 [0.14 0.23]
6mm strong meridian (right eye)	7.60E-17	0.18 [0.14 0.23]
3mm weak meridian (right eye)	2.00E-14	0.16 [0.12 0.20]
3mm weak meridian (left eye)	1.60E-13	0.15 [0.11 0.20]
3mm strong meridian (left eye)	1.30E-12	0.15 [0.11 0.19]
3mm strong meridian (right eye)	1.80E-12	0.15 [0.11 0.19]
Corneal Power (mean of both eyes)	1.10E-13	0.16 [0.11 0.20]

A

B

C

D

Figure 4. Expression analysis of ANGPTL7 Gln175His, Arg177*, and Trp188* in a HEK293 cell line. (A), (B) Western blotting shows intra- and extra-cellular protein levels of ANGPTL7 wild-type, Gln175His, Arg177*, and Trp188*. (C) ELISA was run to quantify intra- and extra-cellular protein levels of ANGPTL7 wild-type, Gln175His, Arg177*, and Trp188* transiently transfected in HEK293 cells (whole-cell lysate was diluted 1:1,000; supernatant was diluted 1:10,000. Both whole-cell lysate and supernatant were normalized against the total amount of protein from the whole-cell lysate); **** = $P < 1 \times 10^{-4}$ statistical difference from WT whole cell lysate; ^ = $P < 0.05$, ^^ = $P < 1 \times 10^{-4}$ statistical difference from WT supernatant. (D) Ratio of secreted versus intracellular ANGPTL7 wild-type, Gln175His, Arg177*, and Trp188* protein levels. Raw ANGPTL7 wild-type, Gln175His, Arg177*, and Trp188* protein levels were normalized to the whole-lysate protein concentration; **** = $P < 1 \times 10^{-4}$ statistical difference from WT. Western blotting and ELISA analysis were repeated on three independent biological replicates. Technical replicates (n=3) were run for the ELISA analysis. P-values were calculated by one-way ANOVA with Tukey's post hoc analysis. Data are represented as mean $\pm$ SEM.

E

Figure 5. ANGPTL7 expression in ocular tissues across species. RNA-sequencing-based expression levels (measured in transcripts per million, TPM) are highest in cornea, trabecular meshwork (TM), and sclera in human (**A**), and African green monkey (**B**) eyes, and in cornea, TM, sclera, optic nerve, and choroid/RPE in C57BL/6J mice; (**C**) *In situ* hybridization (RNAscope) shows *ANGPTL7*/*Angptl7* (red) expression in TM, cornea and sclera in human (**D**) and murine (**E**) eyes. DAPI staining (blue) counterstains cell nuclei. RPE: retinal pigmented epithelium; CB: ciliary body; SC: Schlemm's canal; CM: ciliary muscle; AC: anterior chamber; RGC: retinal ganglion cell; INL: inner nuclear layer; ONL: outer nuclear layer.

Figure 6. Increasing mAngptl7 levels in mouse eyes increases IOP. Murine Angptl7 (mAngptl7) protein was injected into mouse eyes via intravitreal (**A**) or intracameral route (**B**), and IOP was measured over time. After an initial drop, IOP remained elevated for several days in Angptl7-treated eyes compared to PBS treated (CTRL (PBS)) eyes. * = $P < 0.05$; ** = $P < 0.01$ statistical significance compared to PBS treatment. Statistical analyses were performed using Student's *t*-test. Data are presented as means and error bars represent SEM.

Figure 7: Reducing *Angptl7* levels in mice lowers IOP. (A) IOP was significantly lowered in *Angptl7* KO compared to Het and WT mice (mean $\pm$ SEM; **** = $P < 1 \times 10^{-4}$). (B) Intravitreal

injection with 15 μ g of Angptl7-siRNA significantly lowered IOP in two of six siRNAs tested (n=6-8/group) compared to the PBS (n=6) and Naïve (no injection, n=5) groups and remained lowered throughout the end of the study. siRNAs 3 and 5 lowered IOP between 2-4 mmHg starting at week 2 compared to PBS-treated mice. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 1 \times 10^{-3}$, **** = $P < 1 \times 10^{-4}$ statistical difference between siRNA #5 and PBS-treatment. # = $P < 0.05$, ## = $P < 0.01$, ### = $P < 1 \times 10^{-3}$, #### = $P < 1 \times 10^{-4}$ statistical difference between siRNA #3 and PBS-treatment. P-values were calculated using one-way ANOVA with Dunnett's post hoc analysis. (C) qPCR results from micro-dissected limbal ring showed the highest level of knockdown (>50%) of Angptl7 mRNA with siRNAs #3 and #5 compared to PBS-treated mice, which is highly consistent with the IOP lowering observed in mice injected with these two siRNAs (B). Error bars represent SEM.

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