nature portfolio

Peer Review File

Genome-wide meta-analysis identifies 22 loci for normal tension glaucoma with significant overlap with high tension glaucoma

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Summary:
Diaz-Torres et al. report results of their genome-wide association study (GWAS) of normal-tension glaucoma (NTG). The authors performed GWAS analyses in UKBB and CLSA samples, and retrieved GWAS summary statistics from IGGC, FinnGen, and an Asian-ancestry study, and a related quantitative trait, vertical cup-to-disk ratio (VCDR, prior GWAS of UKBB, CLSA and IGGC studies). Results from separate GWAS were combined using standard meta-analyses and multi-trait analysis of GWAS (MTAG) and identified 22 loci (17 novel) associated with NTG. Moreover, the authors compared their NTG loci with prior GWAS results efforts for high-tension glaucoma (HTG), primary open-angle glaucoma (POAG), VCDR and intraocular pressure (IOP), revealed strong genetic overlap between NTG and HTG. They note that 4 loci had different magnitudes of effects between NTG and HTG.

Major Comments:
1. The CLSA study utilized self-report NTG definitions, which are susceptible to misclassification and may lead to inflation/enrichment/exacerbation, particularly in multivariate analyses.
2. Concern with use of non-contemporaneous IOP measure: 'Participants with a diagnosis of glaucoma and IOP under 21 mmHg were considered NTG cases in UKBB and CLSA, provided that the tonography to measure IOP was performed within five years of the glaucoma diagnosis'.
a. Is this widely accepted phenotype definition? If so, where has it been done before?
b. Were results consistent if limited to subjects with IOP within 1 year?
c. Is this 5 years before or after glaucoma diagnosis?
3. REGENIE may provide overly conservative results when study sample size is modest/small and contains a high degree of related subjects, particularly CLSA cohort. It would be helpful if the authors provided Manhattan and Quantile-Quantile (QQ) plots for study specific GWAS and results of meta-analyses.
4. There is no discussion or comment on overlap of loci between MTAG NTG (Supplement Table 1) and joint NTG (Supplement Table 2) – specific loci do not appear to overlap.
5. Concern with meta-analyses and comparison with the VCDR phenotype/data. Did VCDR GWAS summary statistics (n= 282,100, Han et al., 2021) include full subjects from published GWAS or subset to individuals of European-ancestry?
6. Related to above: MTAG was tested using data from subjects of European-ancestry (single ancestral group), however, the authors report analyses with VCDR data, which may have included subjects of non-European – this may violate key assumptions within MTAG and may bias and/or inflated results.
7. Why not use MTAG for joint analysis ('overall sample size of 7,942 cases and 384,431 controls, complemented with 282,100 VCDR values'
8. HTG and NTG are reported to be strongly correlated (rg = 0.84, se = 0.07 p = 5.1e-33), however, the method used to test whether 'different magnitudes of effects' between them does not account for this correlation. The authors are encourage to revise analyses with method that accounts for correlation (e.g., https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1003500#s4).
9. Additional details (provided as a supplement) of COJO results, including locus zoom style plots of raw and post-COJO locus results.

Minor Comments
i. It would be helpful to include genomic position and nearest gene info for Table 1.
ii. Table 1 beta/SE/P-val labels not consistent.
iii. Gene names in figure 1 are very small.
iv. References are duplicated.

Reviewer #2 (Remarks to the Author):

The research paper provides novel insight into independent gene risk loci for 'normal tension glaucoma' (NTG) - the majority (17 of 22) of which overlap with 'high tension glaucoma' (HTG).
The effect size was weaker for NTG than HTG (1.6 fold).
An association is observed between BMP4 and scRNA-seq of RGCs and 'neurons' or 'neuron-like cells' in the NTG cohort - identifying a connection with 'serotonin receptor neurones' (the rotenone induced oxidative stress is not mentioned in the Methods or have a reference). Hard to interpret the relevance to RGCs/glaucoma.
Drug-gene interactions is also explored, and identifies connections with 4 genes of interest.
Surprising that the BMP4 connection to the TGF beta superfamily is not mentioned, given the known role of TGFb in glaucoma.
Comment is made about potential IOP related genes versus 'neurodegenerative' (VCDR) genes in the NTG dataset - further adding to the longstanding discussion/debate about the difference between NTG and HTG, and the use of the word 'notion' in the Introduction could be replaced with 'point of view'.
The gene results reported would seem to support 2 different patho-mechanisms ?
All the genetic statistical analysis in the paper is completely dependent on the quality of data on the input side, ie the Biobank patients recruited. The number of participants is large, which will cover over a lot of 'inaccuracies'. The real question is how clear and accurate can we be that these participants truly have NTG - the paper needs a definition of the criteria used, does it include repeatable visual field loss.
Every patient who attends a glaucoma clinic thinks they have glaucoma, especially if placed on treatment - and it would also be true to say that 20-30% of patients attending glaucoma clinics have OHT (with little or no optic nerve damage), not glaucoma. But as stated earlier this issue is 'corrected' by the sheer volume of patients recruited (though diluting the results).
The earlier descriptions of the UK Biobank was that glaucoma was 'self reported' - has this changed to the ICD 10 criteria ?
The biggest challenge in reviewing the paper was the poorly described role of IOP measurements in the diagnosis of NTG. This was compounded by the use of the word 'tonography' (which is the measurement of the rate of aqueous outflow) when tonometry (measuring the IOP) was the word needed.
'IOP under 21' maybe should read 22 mmHg ?
Difficult to understand the following; 'measure IOP was performed within five years of the glaucoma diagnosis'.
Surely IOP less than 22 mmHG at the time of diagnosis is what is meant ? What's the issue about 5 years ?
The following sentence is unclear also - 'Patients under glaucoma medication or those who had undergone surgery to decrease the IOP at the time of tonography were excluded from the analysis in UKBB and CLSA' - does this mean that some patients were untreated ?

This paper is a very valuable exploration of underlying genetic differences between NTG and HTG, and it would appear that there aren't too many differences in this large database analysis.

Thank you for taking the time to review our manuscript. We appreciate your insightful comments and suggestions. We have carefully considered each of your points and addressed the concerns of the reviewers. In the following pages, we provide a point-to-point answer to each of the comments and explain how we have revised the manuscript accordingly.

Reviewer 1
Major Comments:
1. The CLSA study utilized self-report NTG definitions, which are susceptible to misclassification and may lead to inflation/enrichment/exacerbation, particularly in multivariate analyses.

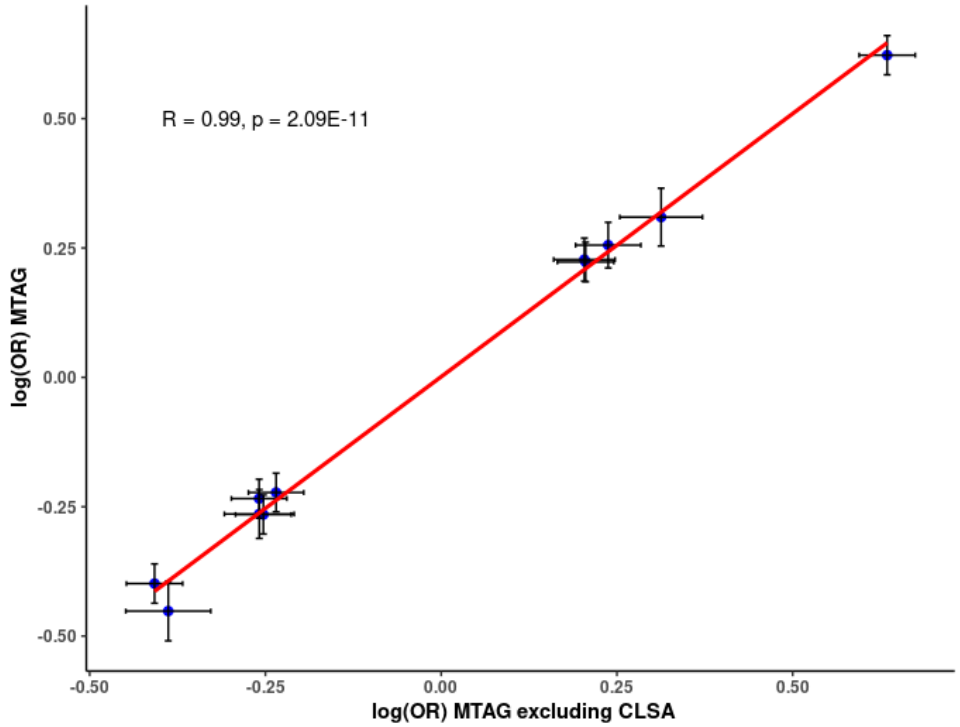
Thanks to the reviewer for the comment. We agree that the self-reported nature of glaucoma can lead to misclassification. We did not aim to use CLSA as a direct estimate for NTG but rather as a highly correlated proxy phenotype that could increase the statistical power to map NTG-related loci through MTAG. MTAG leverages the genetic correlation between correlated traits (i.e., they do not need to be the same trait) to boost the statistical power for the GWAS of each input trait. This is the reason we decided to include this as part of the multi-trait meta-analysis, and is now explained in the Methods section of the manuscript: "***Given that UKB and CLSA cases were not clinically diagnosed with specificity for tension subtypes, we considered them as "probable NTG cases" and used these results as separate traits in the multi-trait meta-analysis.*** " and " *We used Multi-Trait Analysis of GWAS (MTAG), version 2020080, a method for the joint analysis of summary statistics from genome-wide association studies (GWAS) of correlated traits (Turley et al., 2018), and an inverse variance-weighting meta-analysis approach through METAL, version 20211102, to enhance the power for discovering NTG risk loci.* ***MTAG leverages the genetic correlation between correlated traits to boost the statistical power for the GWAS of each input trait****. We conducted a two-stage multi-trait meta-analysis.*

The first stage entailed a meta-analysis using METAL, incorporating NTG probable cases from the UK Biobank (UKB) and the Canadian Longitudinal Study on Aging (CLSA). Subsequently, the results of the UKB and CLSA meta-analysis, vertical cup-disc ratio (VCDR) adjusted for intraocular pressure (IOP), and clinically diagnosed NTG cases of European ancestry from the International Glaucoma Genetics Consortium (IGGC) were integrated into a multi-trait meta-analysis using MTAG. ***This first stage aimed to enhance the statistical power of IGGC NTG cases of European ancestry.***

Loci identified as independently genome-wide significant (P < 5e-8) in the first-stage analysis were validated using an inverse variance-weighting approach in independent datasets (i.e., FinnGen and Asian IGGC). Upon validation of the first-stage results, we jointly analyzed phenotypes that were clinically diagnosed (i.e., IGGC in Europeans and Asians, FinnGen) using METAL and then used MTAG to incorporate the probable NTG cases of UKB and CLSA, and IOP-adjusted VCDR. The second stage resulted in an overall sample size of 7,942 cases and 384,431 controls, with VCDR values available for 97,939 participants. ***Data from UKB and CLSA, and VCDR were used for their high correlation with the clinically diagnosed phenotype, aiming to boost the statistical power of the associations."*** However, given the ambiguous nature of the phenotype, we further tried a leave-one-out approach to ensure that the results of CLSA and UKB were not creating any statistical artifacts. The results remained consistent after excluding the CLSA and UKB meta-analysis, with effect sizes for the independent genome-wide significant loci being highly correlated in an inverse variance-weighted approach; plotted below for the MTAG excluding CLSA, excluding UKB give

a similar result. As expected, the standard errors in the MTAG results that exclude CLSA and UKB were wider, likely implying a higher uncertainty and therefore a lower statistical power. It is also necessary to point out here that the results of the first stage of the meta-analysis, which includes CLSA and UKB, were strongly replicated in FinnGen, which used the ICD10 definition of normal tension glaucoma.

It's important to clarify that the Methods section did not accurately describe the use of METAL and MTAG across the two stages of the meta-analysis, and it has been modified to reflect the information that we are describing here. In the first stage, METAL was employed to conduct a meta-analysis of the UK Biobank (UKB) and Canadian Longitudinal Study on Aging (CLSA) data. In these two cohorts, the aim was to use cases of glaucoma with low IOP as a proxy for NTG, allowing their inclusion in the subsequent MTAG analysis alongside VCDR and IGGC clinically diagnosed NTG in Europeans. In the second stage, METAL was utilized to perform a meta-analysis on clinically diagnosed NTG GWAS datasets (i.e., FinnGen, IGGC Europeans and Asians). Subsequently, MTAG was used to integrate the VCDR, the proxy meta-analysis of UKB and CLSA, and the clinically diagnosed NTG as the main phenotype in the analysis.



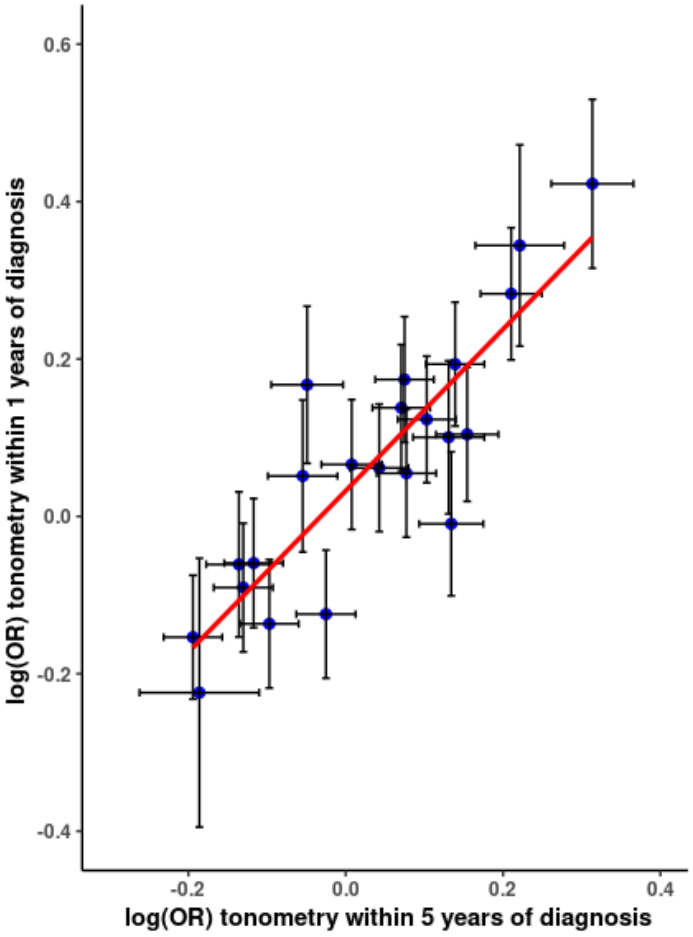
2. Concern with use of non-contemporaneous IOP measure: 'Participants with a diagnosis of glaucoma and IOP under 21 mmHg were considered NTG cases in UKBB and CLSA, provided that the tonography to measure IOP was performed within five years of the glaucoma diagnosis'.
a. Is this widely accepted phenotype definition? If so, where has it been done before?

Thanks to the reviewer for the question. No, this is not a widely accepted definition. It assumes that participants with IOP under 21 mmHg have NTG, but there is a lack of clinical diagnosis based on the ICD-10 criteria for NTG. However as explained in a previous point, the purpose of

using UKB and CLSA data in the first stage of the analysis was not to contribute to a “clean” phenotype but rather to use a proxy highly correlated with NTG that can boost the statistical power for the clinically diagnosed phenotype of NTG; as such we have described this through the manuscript as “probable NTG” rather than NTG. This is also why we excluded FinnGen from the first stage of the meta-analysis, to be able to assess if the NTG results in an independent cohort with clinically diagnosed NTG (FinnGen) were consistent with the results from our MTAG model (containing NTG plus “probable NTG” and a correlated quantitative trait).

b. Were results consistent if limited to subjects with IOP within 1 year?

Thanks to the reviewer for the suggestion. This will decrease the number of “probable NTG” participants by approximately 4.5 fold and therefore conflict with the purpose of the first stage of the analysis, which aims to increase the statistical power for associations based on multiple trait analysis through MTAG. To further show that the results remain consistent while the statistical power decreases, we have now run an analysis on UKB including only participants who had the tonometry performed within 1 year of glaucoma diagnosis, and compared the 22 identified loci with our previous analysis, which was within 5 years. The figure below shows a high correlation but a higher uncertainty of the effect estimate with wider confidence intervals in the "within 1 year" analysis when compared with the "within 5 years" analysis.



c. Is this 5 years before or after glaucoma diagnosis?

We used the maximum IOP measurement within 5 years before diagnosis. Some of the Biobank participants had IOP data from approximately 12 years ago but were only diagnosed with glaucoma 2 years ago; in such cases it is difficult to know if they had high IOP at diagnosis. We selected 5 years as an arbitrary threshold to allow us to select only individuals with at least some data indicating they may have NTG (we have revised this as “probable NTG” in the revised manuscript).

3. REGENIE may provide overly conservative results when study sample size is modest/small and contains a high degree of related subjects, particularly CLSA cohort. It would be helpful if the authors provided Manhattan and Quantile-Quantile (QQ) plots for study specific GWAS and results of meta-analyses.

Thanks for the suggestion. We have now included QQ and Manhattan plots for the GWAS results in the meta-analysis. Please see supplementary Figures 4 to 11.

4. There is no discussion or comment on overlap of loci between MTAG NTG (Supplement Table 1) and joint NTG (Supplement Table 2) – specific loci do not appear to overlap.

Thanks to the reviewer for the comment. We agree that the loci (variant names) in Table 1 (first stage) are not necessarily the same as those in Table 2 (second stage). This is likely due to high LD variants. However, we have further validated the consistency of the effect between loci in the first and second stages of the meta-analysis. As such, we have now included Suppl. Figure 2 and added the following information in the Results section of the manuscript: "**We further validated the consistency of the results between stage one and stage two of the meta-analysis using an IVW approach (r = 0.99, p = 1.7e-11); as per Supplementary Figure 2.**".

5. Concern with meta-analyses and comparison with the VCDR phenotype/data. Did VCDR GWAS summary statistics (n= 282,100, Han et al., 2021) include full subjects from published GWAS or subset to individuals of European-ancestry?

Thanks to the reviewer for the comment. VCDR GWAS data included in this analysis were from individuals of European ancestry. However, it was not clear in the manuscript as the sample size referred to the number of images instead of the number of participants and included images of non-European ancestries. We have now clarified this in the manuscript by including the sample size for individuals of European ancestry and adding the following information to the Methods section: "To identify risk loci specific to NTG, we conducted a large multi-trait meta-analysis of genome-wide association studies (GWAS) across individuals of European and Asian descent. The European ancestry NTG data included the International Genetics of Glaucoma Consortia (IGGC; 3,247 cases and 47,997 controls), UK Biobank (UKB; 2,184 cases and 7,000 controls), Canadian Longitudinal Study on Aging (CLSA; 755 cases and 3,000 controls), FinnGen

(Release 8, 1,756 cases and 326,434 controls), and a structural measurement of the integrity of the optic nerve, **vertical cup-to-disk ratio (VCDR, N = 97,939 participants). VCDR estimates were obtained from UKB and CLSA participants of European ancestry** (Han et al., 2021). The Asian NTG data included a GWAS meta-analysis of 4,418 cases and 34,303 controls from Hong Kong, Singapore, and Japan."

6. Related to above: MTAG was tested using data from subjects of European-ancestry (single ancestral group), however, the authors report analyses with VCDR data, which may have included subjects of non-European – this may violate key assumptions within MTAG and may bias and/or inflated results.

Thanks to the reviewer for the comment. We agree on the importance of including only individuals of a specified ancestry in the MTAG analysis, which is why we added more details to the manuscript, as described in the previous point.

7. Why not use MTAG for joint analysis ('overall sample size of 7,942 cases and 384,431 controls, complemented with 282,100 VCDR values'

The rationale behind this approach was to use MTAG for the first stage of the meta-analysis, where we utilized NTG proxy phenotypes (e.g., CLSA, UKB) and highly correlated traits (VCDR) to boost the statistical power of clinically diagnosed NTG GWAS in IGGC Europeans. We aimed to validate the results in an independent, clinically diagnosed European cohort (FinnGen). Once we were confident in the consistency of the loci, we included FinnGen and clinically diagnosed Asian IGGC data using an inverse variance-weighted method (METAL), accounting for the variance of the effect across different ancestries.

8. HTG and NTG are reported to be strongly correlated (rg = 0.84, se = 0.07 p = 5.1e-33), however, the method used to test whether 'different magnitudes of effects' between them does not account for this correlation. The authors are encourage to revise analyses with method that accounts for correlation (e.g., https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1003500#s4).

Thanks to the reviewer for the suggestions. We agree that when testing for the difference between the effects, if they are independent, then the variance of the difference between the effects is the sum of the variance of each individual one. However, if the effects are correlated, we need to correct for the correlation as well. As such, we have now estimated the difference between the magnitudes of effects in NTG and HTG using the method suggested. These modifications are also included in the manuscript:

"The difference in magnitude of effect between the MTAG NTG results and previously published results for HTG and between FinnGen NTG and HTG was estimated using the equation below. Given the strong correlation between the two phenotypes, we decided to include the method described in Randall et al., which accounted for differences between NTG and HTG

effect estimates β_{htg}, β_{ntg} and corresponding standard errors SE_{htg}, SE_{ntg}using t statistics and adjusting for HTG and NTG correlation, where r was computed as the genetic correlation coefficient across all the loci; effects were estimated on the log(OR) scale:

$$t = \frac{\beta_{htg} - \beta_{ntg}}{\sqrt{SE_{htg}^{2} + SE_{ntg}^{2} - 2r \cdot SE_{htg} \cdot SE_{ntg}}}$$

”

9. Additional details (provided as a supplement) of COJO results, including locus zoom style plots of raw and post-COJO locus results.

Thanks to the reviewer for the suggestion. The plots suggested are now included as supplementary material. See Supplementary Figure 12 to 15. We have also modified the text in the manuscript given that the conditional analysis attenuated the signal of *KPNB1* and *EFCAB13* when conditioning for the *TBKBP1* locus “*The results of the conditional analysis in GCTA-COJO for the top SNP of KPNB1 (rs7220935) and EFCAB13 (rs9894179) suggest that this could be part of the same signal at the TBKBP1 locus, as the signal for TBKBP1 did not remain in the conditional analysis. Similarly, the signal for MIR5580 did not remain after the analysis was conditioned for BMP4, indicating that it is likely the same locus, as shown in Supplementary Figures 12 to 15.*”

Minor Comments

i. It would be helpful to include genomic position and nearest gene info for Table 1.
ii. Table 1 beta/SE/P-val labels not consistent.
iii. Gene names in figure 1 are very small.
iv. References are duplicated.

Thanks to the reviewer for the thoughtful suggestions. We have addressed all of the comments and modified the manuscript accordingly.

Reviewers 2

1. An association is observed between BMP4 and scRNA-seq of RGCs and 'neurons' or 'neuron-like cells' in the NTG cohort - identifying a connection with 'serotonin receptor neurones' (the rotenone induced oxidative stress is not mentioned in the Methods or have a reference). Hard to interpret the relevance to RGCs/glaucoma.

Thanks to the reviewer for the comment. We have now included the information relevant to the reference in the Methods section. Rotenone-induced oxidative stress is often used to model neurodegenerative diseases. Given that NTG is believed to have a significant neurodegenerative component, we utilized scRNA-seq data from RGCs and neuron-like cells, including those exposed to rotenone-induced oxidative stress. This approach helps us identify genes associated with RGCs and neuron-like cells (e.g., serotonin receptor neurons), highlighting genes expressed under neurodegenerative stressors that could potentially be linked to NTG etiology. However, we agree that interpreting these findings is challenging, and further validation and investigation of the mechanisms behind this interaction are necessary.

2. Drug-gene interactions is also explored, and identifies connections with 4 genes of interest. Surprising that the BMP4 connection to the TGF beta superfamily is not mentioned, given the known role of TGFb in glaucoma.

Thanks to the reviewer for the suggestion, we are now including the connection of BMP4 to TGFb in the Discussion of the manuscript " **Bone Morphogenetic Protein 4 (*BMP4*) has been associated with retinal ganglion cell integrity in mouse models. There is a well-known interaction between BMP4 and TGF-β that could play a significant role in NTG pathogenesis through effects on the trabecular meshwork and optic nerve head. TGF-β2 is significantly upregulated in patients with glaucoma[39], and increased concentrations of TGF-β have been observed in the aqueous humor and reactive optic nerve astrocytes of individuals diagnosed with POAG. It has also been suggested that BMP4 acts as an endogenous inhibitor of TGF-β2 within the human trabecular meshwork and aids in controlling the increased accumulation of extracellular matrix protein deposits[42], thereby maintaining IOP within normal limits**."

3. Comment is made about potential IOP related genes versus 'neurodegenerative' (VCDR) genes in the NTG dataset - further adding to the longstanding discussion/debate about the difference between NTG and HTG, and the use of the word 'notion' in the Introduction could be replaced with 'point of view'. The gene results reported would seem to support 2 different patho-mechanisms ?

Thanks for the question. We have further clarified our point of view on the two different pathomechanisms in the Discussion. "**Despite the high genetic overlap, we have shown that IOP-related loci tend to have smaller effect sizes in NTG compared to HTG. However, loci that are independent of IOP have similar effect sizes on NTG and HTG. This means that even if there are shared mechanisms leading to the development of the major subtypes of primary open-angle glaucoma, there could be some genetic profiles that are more sensitive to the ocular hypertension process. However, the fact that NTG has some specific loci not associated at a genome-wide significant level with the high-pressure subtype could potentially imply the presence of specific mechanisms that are more neurodegenerative in**

nature, that leads to the ocular neuropathy and that is exacerbated by a comorbid increase in ocular pressure."

4. All the genetic statistical analysis in the paper is completely dependent on the quality of data on the input side, ie the Biobank patients recruited. The number of participants is large, which will cover over a lot of 'inaccuracies'. The real question is how clear and accurate can we be that these participants truly have NTG - the paper needs a definition of the criteria used, does it include repeatable visual field loss. Every patient who attends a glaucoma clinic thinks they have glaucoma, especially if placed on treatment - and it would also be true to say that 20-30% of patients attending glaucoma clinics have OHT (with little or no optic nerve damage), not glaucoma. But as stated earlier this issue is 'corrected' by the sheer volume of patients recruited (though diluting the results).

Thanks to the reviewer for this comment. We agree that the accuracy of the phenotype is highly important and that inconsistencies in phenotype definition or measurement could lead to inaccuracies that result in misleading results. With this in mind, we have used NTG cases that were diagnosed according to the ICD-10 criteria for normotensive glaucoma. This includes reviewed and published data from the International Glaucoma Genetics Consortium and FinnGen. Data from the UK Biobank and CLSA were used as proxies for NTG, and given their correlation with the clinical phenotype, were utilized in MTAG to boost the statistical power of the associations. MTAG leverages the genetic correlation between correlated traits (i.e., they do not need to be the same trait) to boost the statistical power for the GWAS of each input trait. This is the reason we decided to include this as part of the multi-trait meta-analysis, as is now explained in the Methods section of the manuscript: "***Given that UKB and CLSA cases were not clinically diagnosed with specificity for tension subtypes, we considered them as "probable NTG cases" and used these results as separate traits in the multi-trait meta-analysis.*** " and " *We used Multi-Trait Analysis of GWAS (MTAG), version 2020080, a method for the joint analysis of summary statistics from genome-wide association studies (GWAS) of correlated traits (Turley et al., 2018), and an inverse variance-weighting meta-analysis approach through METAL, version 20211102, to enhance the power for discovering NTG risk loci.* ***MTAG leverages the genetic correlation between correlated traits to boost the statistical power for the GWAS of each input trait****. We conducted a two-stage multi-trait meta-analysis.*

5. The earlier descriptions of the UK Biobank was that glaucoma was 'self reported' - has this changed to the ICD 10 criteria ?

Thanks for the question, UK Biobank data at present includes both ICD 10 criteria and self-reported glaucoma. In our study, glaucoma was 'self-reported' in CLSA as for UKB; we used the ICD10 for glaucoma, which is not normal tension glaucoma-specific. As described in the manuscript "***Glaucoma was self-reported in CLSA following diagnosis by a clinician as per the report in the item 'ICQ_GLAUC_COM' (Has a doctor ever told you that you have glaucoma?)***

*[14,15]. **UK Biobank was restricted to glaucoma diagnosed under the International Classification of Diseases (ICD) 10 criteria***, without specific classification for HTG or NTG. Thus, p*articipants with a diagnosis of glaucoma and IOP under 21 mmHg were considered NTG cases in UKB and CLSA, provided that the tonometry to measure IOP was performed within five years of the glaucoma diagnosis. Maximum IOP was used when multiple measurements were present. Patients receiving glaucoma medication or those who had undergone surgery to decrease the IOP at the time of the tonometry were excluded from the analysis in UKB and CLSA. Controls were randomly selected from UKB and CLSA datasets, maintaining a ratio of 1:3, from a pool of individuals who had no reported ocular conditions. **Given that UKB and CLSA cases were not clinically diagnosed with specificity for tension subtypes, we considered them as "probable NTG cases" and used these results as separate traits in the multi-trait meta-analysis***."

6. The biggest challenge in reviewing the paper was the poorly described role of IOP measurements in the diagnosis of NTG. This was compounded by the use of the word 'tonography' (which is the measurement of the rate of aqueous outflow) when tonometry (measuring the IOP) was the word needed.
'IOP under 21' maybe should read 22 mmHg ?

Thanks to the reviewer for the comment. We agree that there is a misuse of the word "tonography" where we were referring to the measuring of IOP, i.e., tonometry. As such, we have now modified the manuscript accordingly." *Participants with a diagnosis of glaucoma and IOP under 21 mmHg were considered NTG cases in UKB and CLSA, provided that the **tonometry** to measure IOP was performed within five years of the glaucoma diagnosis. Maximum IOP was used when multiple measurements were present. Patients receiving glaucoma medication or those who had undergone surgery to decrease the IOP at the time of the **tonometry** were excluded from the analysis in UKB and CLSA. "*

7. Difficult to understand the following; 'measure IOP was performed within five years of the glaucoma diagnosis'. Surely IOP less than 22 mmHG at the time of diagnosis is what is meant ? What's the issue about 5 years ?

Some of the UK Biobank participants had IOP data from say 12 years ago but were only diagnosed with glaucoma 2 years ago; in such cases it is difficult to know if they had high IOP at diagnosis. We selected 5 years as an arbitrary threshold to allow us to select only individuals with at least some data indicating they may have NTG (we have revised this as "probable NTG" in the revised manuscript. The 5 years is arbitrary and a tighter threshold may have been better although as discussed in our response to Reviewer 1, using a tight threshold greatly reduces the number of "probable NTG" cases, which diminishes the power of our MTAG model (the MTAG approach allows us to harness the correlation between "probable NTG" and NTG to increase power for our genetic analysis).

8. The following sentence is unclear also - 'Patients under glaucoma medication or those who had undergone surgery to decrease the IOP at the time of tonography were excluded from the analysis in UKBB and CLSA' - does this mean that some patients were untreated ?

In our study, when analyzing the IOP measurements from the UKB and CLSA, we specifically considered the participants' medication status at the time of IOP measurement, not at their initial diagnosis of glaucoma. The time points for glaucoma diagnosis and IOP measurements do not overlap. Therefore, this does not imply that the patients were untreated for glaucoma; rather, they were likely undiagnosed at the time of the tonometry.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Authors were responsive to critiques.

Minor comment: Suggestion to include figure provide in author response for comment 2b (b. Were results consistent if limited to subjects with IOP within 1 year?) in the supplemeent.

Reviewer #3 (Remarks to the Author):

In the revised manuscript, Diaz-Torres et al. describe their efforts to identify glaucoma loci that are specifically associated with normal tension glaucoma (NTG). In responding to the points raised during previous reviews, the authors have substantially revised their manuscript.

This is a well-written and interesting manuscript describing a large genome-wide association study (GWAS) of high clinical relevance. The genetic and biological factors distinguishing NTG and primary open-angle glaucoma (POAG) remain poorly understood, and it is unclear whether the same pathomechanisms are involved in the development of these conditions. This manuscript provides insight into the genetic commonalities and differences in patients receiving these two clinical diagnoses.

The authors have responded well to the majority of concerns raised by the reviewers. Yet, one concern remains: much of the discussion in the previous review centered around the definition of NTG cases in the UK Biobank and the Canadian Longitudinal Study on Aging (CLSA). A specific point of concern was the reliance on intraocular pressure (IOP) measurements made as much as five years before or after the glaucoma or NTG diagnosis. Although the authors have clarified their inclusion criteria, this remains a concern. Concerns about misclassification were further amplified by the fact that glaucoma was self-reported by the patients. These concerns are partly addressed by the authors' two-stage approach to analysis, which is now well described.

Furthermore, an argument can be made that misclassification of patients would lead to heterogeneity within the sample population, an increase in genetic diversity, and a loss of statistical significance. This approach is more likely to generate false negatives than false positives. Consequently, additional loci associated with NTG may exist, but those reported herein are likely to be truly associated with NTG in these populations.

Yet, this approach remains a shortcoming of the study and one that the investigators cannot fully address. The Discussion section would benefit from the inclusion of a short paragraph highlighting this limitation of the study.

Thank you for taking the time to review our manuscript. We appreciate your insightful comments and suggestions. We have carefully considered each of your points and addressed the concerns of the reviewers. In the following pages, we provide a point-to-point answer to each of the comments and explain how we have revised the manuscript accordingly.

Reviewer 1

Minor comment: Suggestion to include figure provide in author response for comment 2b (b. Were results consistent if limited to subjects with IOP within 1 year?) in the supplemeent.

Thanks for the suggestions. As proposed, we have now added the figure provided for comment 2b as Supplementary Figure 16 for the study. Furthermore, we have also included the following description in the manuscript: "**However, to ensure consistency in results when IOP measurements are taken closer to the time of glaucoma diagnosis, we re-ran the analysis to include participants who had tonometry within one year of their diagnosis. The results were consistent, but the power decreased due to a reduction in the number of 'probable NTG' participants by approximately 4.5-fold, as shown in Supplementary Figure 16.** "

Reviewer 3

The authors have responded well to the majority of concerns raised by the reviewers. Yet, one concern remains: much of the discussion in the previous review centered around the definition of NTG cases in the UK Biobank and the Canadian Longitudinal Study on Aging (CLSA). A specific point of concern was the reliance on intraocular pressure (IOP) measurements made as much as five years before or after the glaucoma or NTG diagnosis. Although the authors have clarified their inclusion criteria, this remains a concern. Concerns about misclassification were further amplified by the fact that glaucoma was self-reported by the patients. These concerns are partly addressed by the authors' two-stage approach to analysis, which is now well described.

Furthermore, an argument can be made that misclassification of patients would lead to heterogeneity within the sample population, an increase in genetic diversity, and a loss of statistical significance. This approach is more likely to generate false negatives than false positives. Consequently, additional loci associated with NTG may exist, but those reported herein are likely to be truly associated with NTG in these populations.

Yet, this approach remains a shortcoming of the study and one that the investigators cannot fully address. The Discussion section would benefit from the inclusion of a short paragraph highlighting this limitation of the study.

Thanks to the reviewer for the suggestion. We have now included the following information in the discussion as a limitation of the study: "**A limitation of our study is the reliance on IOP measurements, which included participants who had tonometry up to five years before or after the diagnosis of glaucoma. This introduces a risk of misclassification, particularly given that glaucoma diagnoses were self-reported in CLSA. While our two-stage approach to analysis mitigates some of these concerns by reducing the likelihood of false positives, it may increase the heterogeneity of the sample and the potential for false negatives. Consequently, while the loci identified in this study are likely robust, additional loci associated with NTG may remain undetected.**"