New loci associated with central cornea thickness include \textit{COL5A1}, \textit{AKAP13} and \textit{AVGR8}

Veronique Vitart\textsuperscript{1,*}, Goran Benčić\textsuperscript{2}, Caroline Hayward\textsuperscript{1}, Jelena Škunca Herman\textsuperscript{2}, Jennifer Huffman\textsuperscript{1}, Susan Campbell\textsuperscript{1}, Kajo Bučan\textsuperscript{3}, Pau Navarro\textsuperscript{1}, Grgo Gunjaca\textsuperscript{3}, Josipa Marin\textsuperscript{3}, Lina Zgaga\textsuperscript{5,6}, Ivana Kolčić\textsuperscript{6}, Ozren Polašek\textsuperscript{5,6}, Mirna Kirin\textsuperscript{5}, Nicholas D. Hastie\textsuperscript{1}, James F. Wilson\textsuperscript{5}, Igor Rudan\textsuperscript{4,5}, Harry Campbell\textsuperscript{5}, Zoran Vatavuk\textsuperscript{2}, Brian Fleck\textsuperscript{7} and Alan Wright\textsuperscript{1}

\textsuperscript{1}MRC Human Genetics Unit, IGMM, Edinburgh, UK, \textsuperscript{2}Department of Ophthalmology, Hospital ‘Sestre Milosrdnice’, Zagreb, Croatia, \textsuperscript{3}Faculty of Medicine, University of Split, Croatia, \textsuperscript{4}Croatian Centre for Global Health, University of Split Medical School, Croatia, \textsuperscript{5}Centre for Population Health Sciences, University of Edinburgh, Edinburgh, UK, \textsuperscript{6}Andrija Stampar School of Public Health, Zagreb University, Croatia and \textsuperscript{7}Princess Alexandra Eye Pavilion, Edinburgh, UK

Received July 3, 2010; Revised and Accepted August 11, 2010

Central corneal thickness (CCT) is a highly heritable trait, which has been proposed to influence disorders of the anterior segment of the eye. A genome-wide association study (GWAS) of CCT was performed in 2269 individuals from three Croatian and one Scottish population. In the discovery set (1445 individuals), two genome-wide significant associations were identified for single nucleotide polymorphisms rs12447690 ($\beta = 0.23$ SD, $P = 4.4 \times 10^{-9}$) and rs1536482 ($\beta = 0.22$ SD, $P = 7.1 \times 10^{-8}$) for which the closest candidate genes (although \textgtrsim 90 kb away) were zinc finger 469 (\textit{ZNF469}) on 16q24.2 and collagen 5 alpha 1 (\textit{COL5A1}) on 9q34.2, respectively. Only the \textit{ZNF469} association was confirmed in our replication set (824 individuals, $P = 8.0 \times 10^{-4}$) but \textit{COL5A1} remained a suggestive association in the combined sample ($\beta = 0.16$ SD, $P = 1.1 \times 10^{-6}$). Following a larger meta-analysis including recently published CCT GWAS summary data, \textit{COL5A1} was genome-wide significant ($\beta = 0.13$ SD, $P = 5.1 \times 10^{-8}$), together with two additional novel loci. The second new locus (defined by rs1034200) was 5 kb from the \textit{AVGR8} gene, encoding a putative transcription factor with typical ZNF and KRAB domains, in chromosomal region 13q12.11 ($\beta = 0.14$ SD, $P = 3.5 \times 10^{-8}$). The third new locus (rs6496932), on 15q25.3 ($\beta = 0.13$, $P = 1.4 \times 10^{-6}$), was within a wide linkage disequilibrium block extending into the 5′ end of the \textit{AKAP13} gene, encoding a scaffold protein concerned with signal transduction from the cell surface. These associations offer mechanistic insights into the regulation of CCT and offer new candidate genes for susceptibility to common disorders in which CCT has been implicated, including primary open-angle glaucoma and keratoconus.

INTRODUCTION

The cornea is a transparent connective tissue in which the stroma constitutes over 90% of its thickness and is composed of collagen fibrils arrayed in a lamellar architecture (1). Corneal thickness is a determinant of corneal refractive power which contributes to normal vision and has been implicated in a number of disorders affecting the anterior segment of the eye. Central corneal thickness (CCT) has been associated with progression from ocular hypertension to primary open-angle glaucoma (POAG) (2,3), although it is not consistently correlated with POAG within families (4). Extreme thinning of the cornea characterizes the rare autosomal recessive disorder brittle cornea syndrome (BCS), which is also associated with systemic features particularly affecting skin, teeth, joints and hair (5). Reduced corneal thickness has also been reported in
other connective tissue disorders, including Ehlers–Danlos syndrome (EDS) types I, II and VI and osteogenesis imperfecta, in which the underlying disorder involves the disruption of collagen fibril assembly (6–8). Keratoconus is a more common condition in which thinning of the cornea is associated with weakening of the collagenous structure of the cornea (9).

Heritability is a measure of the extent to which additive genetic variation contributes to the trait variability and is reported to be uniformly high for corneal thickness, with estimates ranging from 0.71 to 0.95, across studies of European as well as Asian heritage (4,10–12). In our preliminary description of CCT in adult population samples from the Croatian islands of Vis and Korčula, which was measured along with five other oculometric traits, CCT stood out as being remarkably uninfluenced by age, gender, BMI and height (11).

The recent publication of a genome-wide association study (GWAS) performed on five cohorts of Australian and UK descent for this trait revealed two genome-wide significant signals at 16q24.2 and 13q14.1, both in intergenic regions, putatively influencing, respectively, the genes ZNF469 and FOXO1 (13). Zinc finger 469 (ZNF469) is a poorly characterized member of the large family of genes encoding ZNF proteins in which deleterious coding region mutations were described in BCS families, highlighting its fundamental role in corneal organization (5). Here we present GWAS results for CCT carried out in 2269 individuals from the two Croatian island populations (11) complemented by an urban Croatian population sample (Split) and a Scottish island population sample (Orkney). We then performed a meta-analysis using our combined four population data together with published summary data from five other population samples, all of UK descent, analysed by Lu et al. (13).

RESULTS

Genome-wide association study

The results of the initial association scan performed in the discovery set of CROATIA-Vis and Korčula samples \(n = 1445\), using only genotyped data, are presented in Figure 1. The samples were genotyped using 275 001 single nucleotide polymorphisms (SNPs) that were shared between the two populations and tested for association by meta-analysis, with a genome-wide significance threshold, based on Bonferroni’s correction, of \(1.8 \times 10^{-7}\). Two markers reached genome-wide significance; first, rs12447690 on chromosome 16q24.2 \((\beta = 0.23\) standard deviation (SD), \(P = 4.4 \times 10^{-10}\)), the same SNP recently reported to be associated with CCT by Lu et al. (13). The SNP is located 165.7 kb 5′ of the ZNF469 gene and 187.2 kb 3′ of BANP. The second genome-wide significant SNP, which has not previously been associated with CCT, was rs1536482 in chromosomal region 9q34.2 \((\beta = 0.22\) SD, \(P = 7.1 \times 10^{-8}\)), situated 93.1 kb 5′ of the collagen type V, alpha1 chain gene COL5A1 and 108.1 kb 3′ of the RXRA gene, encoding the retinoic X receptor alpha. The COL5A1 gene is a strong \textit{a priori} candidate gene for this association since it is mutated in EDS types I and II patients and is an essential component of stromal collagen (1,14,15).

The third most significant SNP, rs11694554 in chromosomal region 2q36.3, was only nominally significant \((\beta = 0.27\) SD, \(P = 1.7 \times 10^{-6}\)), although it is located within an intron of another biologically relevant candidate gene, COL4A3, encoding collagen type IV, alpha3 chain, in which polymorphisms have recently been associated with keratoconus in a Slovenian cohort (16).

We then sought to confirm the above associations in a replication sample from the urban Croatian population of Split and from the Scottish islands of Orkney \(n = 824\). This GWAS confirmed the signal near ZNF469 on chromosome 16 \((\beta = 0.18\) SD, \(P = 8.0 \times 10^{-4}\)). Each of the four population samples showed comparable effect size and direction of effect, with each rs12447690 G allele decreasing CCT by 0.21 SD in a weighted correction, of 1.8 × 10⁻⁷. Two markers reached genome-wide significance; first, rs12447690 on chromosome 16q24.2 (β = 0.23 standard deviation (SD), P = 4.4 × 10⁻¹⁰), the same SNP recently reported to be associated with CCT by Lu et al. (13). The SNP is located 165.7 kb 5′ of the ZNF469 gene and 187.2 kb 3′ of BANP. The second genome-wide significant SNP, which has not previously been associated with CCT, was rs1536482 in chromosomal region 9q34.2 (β = 0.22 SD, P = 7.1 × 10⁻⁸), situated 93.1 kb 5′ of the collagen type V, alpha1 chain gene COL5A1 and 108.1 kb 3′ of the RXRA gene, encoding the retinoic X receptor alpha. The COL5A1 gene is a strong a priori candidate gene for this association since it is mutated in EDS types I and II patients and is an essential component of stromal collagen (1,14,15).

The third most significant SNP, rs11694554 in chromosomal region 2q36.3, was only nominally significant (β = 0.27 SD, P = 1.7 × 10⁻⁶), although it is located within an intron of another biologically relevant candidate gene, COL4A3, encoding collagen type IV, alpha3 chain, in which polymorphisms have recently been associated with keratoconus in a Slovenian cohort (16).

We then sought to confirm the above associations in a replication sample from the urban Croatian population of Split and from the Scottish islands of Orkney (n = 824). This GWAS confirmed the signal near ZNF469 on chromosome 16 (β = 0.18 SD, P = 8.0 × 10⁻⁴). Each of the four population samples showed comparable effect size and direction of effect, with each rs12447690 G allele decreasing CCT by 0.21 SD in a weighted average (Table 1 and Fig. 2). The signal was consistent across populations (heterogeneity I² = 0%, P = 0.67 for test of heterogeneity) and explained 1.4, 3.4, 1.5 and 1.4% of the trait variance, respectively in Vis, Korčula, Split and Orkney. In contrast, the effects associated with the COL5A1 and COL4A3 collagen genes were not uniform across populations (Table 1 and Fig. 2), with the P-value for heterogeneity either close to significant (P = 0.06, I² = 58.9%) for rs1536482 (near COL5A1) or significant, for rs11694554 (in COL4A3) with P = 0.0095 and I² = 73.8%. Reflecting these heterogeneity values, the combined four-population meta-analysis P-values increased to P = 2.6 × 10⁻¹¹ for rs12447690 (near ZNF469), whereas they were
<table>
<thead>
<tr>
<th>Study</th>
<th>Corneal Thickness, mm</th>
<th>Frequency</th>
<th>b (SE)</th>
<th>P</th>
<th>Frequency</th>
<th>b (SE)</th>
<th>P</th>
<th>Frequency</th>
<th>b (SE)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CROATIA_Vis_island</td>
<td>596</td>
<td>445–670</td>
<td>561.2 (34.6)</td>
<td>0.40</td>
<td>–0.38 (0.11)</td>
<td>0.18</td>
<td>0.003</td>
<td>0.28 (0.07)</td>
<td>0.30 (0.12)</td>
<td>0.08</td>
</tr>
<tr>
<td>CROATIA_Korcula_island</td>
<td>849</td>
<td>457–700</td>
<td>555.6</td>
<td>0.38</td>
<td>–0.21 (0.11)</td>
<td>0.03</td>
<td>0.10</td>
<td>–0.30 (0.07)</td>
<td>1.05</td>
<td>0.05</td>
</tr>
<tr>
<td>CROATIA_Split</td>
<td>349</td>
<td>457–662</td>
<td>561 (36.3)</td>
<td>0.36</td>
<td>–0.38 (0.11)</td>
<td>0.03</td>
<td>0.10</td>
<td>–0.03 (0.12)</td>
<td>0.8</td>
<td>0.08</td>
</tr>
<tr>
<td>ORCADES (UK)</td>
<td>475</td>
<td>430–668</td>
<td>536 (33.4)</td>
<td>0.29</td>
<td>–0.38 (0.11)</td>
<td>0.03</td>
<td>0.10</td>
<td>–0.30 (0.07)</td>
<td>1.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\( b \) is the size of the coded allele’s additive effect, given in SD of the trait adjusted for age and ancestry principal components; SE, standard error of the estimate; \( P \) is the \( P \)-value for the association.

### Meta-analysis including published CCT GWAS data

In order to identify further QTL associations to CCT we performed a meta-analysis of our combined four population data with UK/Australian summary data of suggestive SNPs published by Lu et al. (13) on CCT measured in 5058 individuals. Three independent SNPs passed the genome-wide significance threshold (\( P = 5 \times 10^{-8} \)) in addition to the ZNF469 region (rs12447690) on 16q24.2 and the FOXO1 region (rs2755237) previously reported (Fig. 4). The first was rs1034200 on 13q12.11 (\( \beta = 0.14 \) SD, \( P = 3.5 \times 10^{-8} \)). The second was rs10471977 on 5q11.2, at rs10471977, with a \( P \)-value very close to genome-wide significance (\( P = 3.71 \times 10^{-7} \)), despite the lowering allele being relatively rare [minor allele frequency (MAF) 0.05–0.09]. This resulted from its high mean effect size [0.3 SD with 95% CI (0.41; 0.18)] and low heterogeneity across studies (\( I^2 = 21\% \)), \( P \)-value for heterogeneity 0.38) (Fig. 2). The proportion of CCT variance explained by this SNP was 2.18, 1.17, 3.1 and 0.3%, respectively, for the Vis, Korčula, Split and Orkney samples. The results were fully consistent with an additive model for rs12447690 and rs1536482 but this was less clear for rs10471977 due to the low number of homozygous genotypes for the minor allele (Supplementary Material, Fig. S1).

Analysis of imputed data did not reveal any more strongly associated SNPs beyond the linkage disequilibrium (LD) blocks defined by the associations found using genotyped SNPs (Supplementary Material, Table S1). The resulting quantile–quantile plot was consistent with an excess of true genetic associations, with modest genomic control inflation for each population (inflation factor, \( \lambda = 1.0 \) for Vis, 1.01 for Korčula, 1.02 for Split and 1.04 for Orkney), suggesting that the observed results were not due to population stratification (Supplementary Material, Fig. S2). Association profiles for the top-three most significantly associated regions, on 16q24.2, 5q11.2 and 9q34.2, and colour-coded LD strength between tested SNP and the most significant genotyped SNPs (Supplementary Material, Table S1). The resulting blocks defined by the associations found using genotyped SNPs (Supplementary Material, Table S1).

Meta-analysis including published CCT GWAS data

In order to identify further QTL associations to CCT we performed a meta-analysis of our combined four population data with UK/Australian summary data of suggestive SNPs published by Lu et al. (13) on CCT measured in 5058 individuals. Three independent SNPs passed the genome-wide significance threshold (\( P = 5 \times 10^{-8} \)) in addition to the ZNF469 region (rs12447690) on 16q24.2 and the FOXO1 region (rs2755237) previously reported (Fig. 4). The first was rs1034200 on 13q12.11 (\( \beta = 0.14 \) SD, \( P = 3.5 \times 10^{-8} \)). The second was rs10471977 on 5q11.2, at rs10471977, with a \( P \)-value very close to genome-wide significance (\( P = 3.71 \times 10^{-7} \)), despite the lowering allele being relatively rare [minor allele frequency (MAF) 0.05–0.09]. This resulted from its high mean effect size [0.3 SD with 95% CI (0.41; 0.18)] and low heterogeneity across studies (\( I^2 = 21\% \)), \( P \)-value for heterogeneity 0.38) (Fig. 2). The proportion of CCT variance explained by this SNP was 2.18, 1.17, 3.1 and 0.3%, respectively, for the Vis, Korčula, Split and Orkney samples. The results were fully consistent with an additive model for rs12447690 and rs1536482 but this was less clear for rs10471977 due to the low number of homozygous genotypes for the minor allele (Supplementary Material, Fig. S1).

Analysis of imputed data did not reveal any more strongly associated SNPs beyond the linkage disequilibrium (LD) blocks defined by the associations found using genotyped SNPs (Supplementary Material, Table S1). The resulting quantile–quantile plot was consistent with an excess of true genetic associations, with modest genomic control inflation for each population (inflation factor, \( \lambda = 1.0 \) for Vis, 1.01 for Korčula, 1.02 for Split and 1.04 for Orkney), suggesting that the observed results were not due to population stratification (Supplementary Material, Fig. S2). Association profiles for the top-three most significantly associated regions, on 16q24.2, 5q11.2 and 9q34.2, and colour-coded LD strength between tested SNP and the most significant genotyped SNPs are represented in their genomic context in Figure 3. The top hits for each of these regions were rs12447690 with \( P = 1.13 \times 10^{-11} \) on 16q24.2, rs3118520 with \( P = 2.66 \times 10^{-7} \) on 9q34.2 and rs10056675 with \( P \)-value = \( 2.56 \times 10^{-7} \) on 5q11.2 (Supplementary Material, Table S1).
The second genome-wide significant SNP associated with CCT was rs6496932 on 15q25.3 ($\beta = 0.13$ SD, $P = 1.4 \times 10^{-8}$; $\beta = 0.12$ SD, $P = 10^{-8}$ in our combined four populations alone), which is located between the Phosphodiesterase 8A (PDE8A) gene and A-kinase Anchor Protein 13 (AKAP13) gene, both of which are thought to be involved in signal transduction. The associated LD block was close to 126 kb and encompassed the 5’ end of the AKAP13 gene. The minor (C) allele frequency was 0.22.

The third genome-wide significant SNP associated with CCT was rs7044529 on 9q34.3 ($\beta = 0.13$ SD, $P = 5.1 \times 10^{-8}$; $\beta = 0.10$ SD, $P = 1.9 \times 10^{-8}$ in our combined four populations alone), which is within an intron of the COL5A1 gene, 127.5 kb from the signal observed in the UK/Croatian GWAS and located within another LD block (pairwise LD measures between each SNP were very low: $D' = 0.006$, $r^2 = 0$). Unlike the ZNF469 signal, the FOXO1 signal, designated by SNP rs2755237, which is upstream of the Forkhead Box 01 (FOXO1) gene in chromosomal region 13q14.1, was not genome-wide significant in our independent study alone with a combined meta-analysis $P$-value of 0.01. However, the effect was in the same direction in the UK/Croatian study populations as reported by Lu et al. (13), although smaller in effect size (A allele: $\beta = 0.14$, 0.06, 0.14 and 0.18 SD in Vis, Korčula, Split and Orkney, respectively).

**DISCUSSION**

This study confirmed, refined and discovered further components of the complex genetic architecture that is emerging underlying natural variation in corneal thickness. The major finding was the identification of three new genome-wide significant loci associated with CCT, in the region of the COL5A1, AVGR8 and AKAP13 genes, respectively. A QTL (defined by rs1536482) upstream of COL5A1 was initially found to be genome-wide significant in our discovery set GWAS analysis of population samples from two Croatian islands (Vis, Korčula) and interestingly Lu et al. (13) reported an SNP (rs7044529) in a different LD block, within an intron of the COL5A1 gene, with suggestive association in their cohorts. The meta-analysis of their hits with our results did generate a genome-wide significant $P$-value ($5 \times 10^{-8}$) for this latter SNP. The fact that the two signals were not in the same LD block, and the heterogeneity of the associations we observed in our populations (stronger signals in Vis and Korčula), could be due to the same causal signal with different LD patterns in the different populations. While this is unlikely for a common SNP, given the strong match of pairwise LD between the Croatian isolate and the CEU reference populations that we have reported (17), it could occur if a rarer SNP or more likely a series of rare variants of strong effect arose independently on different haplotypes. Larger meta-analyses would be necessary to test in the future whether the two signals are, alternatively, due to two independent common variants. Re-sequencing projects focusing on the extremes of the CCT distribution and taking advantage of the relatedness of participants in the Croatian and Orkney populations would also help fine mapping causal variants if these are rare.

There is a large body of evidence to support COL5A1, and to a lesser extent COL4A3, as candidates for regulating CCT. Collagen type V has been shown by immuno-electron microscopy to be expressed in the cornea (1,18), and the ratio of collagen V to other fibrillar collagens is particularly high (15–20%) in this tissue (19). *In vitro* fibrillogenesis experiments have shown a direct relationship between the ratio of collagen V to collagen I and fibril diameter (19). Type V collagens buried within the more structural collagen I fibrils in the corneal stroma are thought to restrict the diameter of these fibrils (18,20). Col5a1 haploinsufficient mouse models of classical EDS have shown that type V collagen is a key regulator of fibril nucleation in the corneal stroma, which is on average 25% thinner in a heterozygous Col5a1 null mouse cornea than wild-type and has fewer collagen fibrils, with most showing increased diameter (21,22). This region is therefore a candidate for finding a synthetic association (23), since rare mutations in COL5A1 causing corneal...
thinning are already known to exist in EDS patients (https://eds.gene.le.ac.uk/variants_statistics.php).

The second genome-wide significant locus is on 13q12.3, close to a predicted KRAB domain-containing ZNF transcription factor (AVGR8). The function of this locus is completely unknown. However, it is clear that tight regulation of gene expression is likely to be necessary to achieve the correct assembly and exquisitely structured organization of the cornea (14). Only a few genes regulating corneal gene expression are known, including some in which deleterious mutations cause corneal thinning or thickening. These include the paired-like homeo-domain containing VSX1 and ZNF transcription factor genes, ZNF469, in which mutations can cause BCS (5), and TCF8 (for which COL4A3 is a regulated target). ZNF transcription factors represent a large protein family but only a few have known targets and functions. The AVGR8-associated QTL on 13q12.11 could thus add an extra player to the regulatory network underlying corneal development.

The third novel genome-wide association was located within a wide LD block that extends into the 5′ end of the AKAP13 gene in 15q25.3. The AKAP13-encoded adaptor protein is thought to couple signals from cell surface receptors to transcription factors via Rho signalling and there is a binding site for the regulatory transcription factor FOXF2 in its promoter. In the gut, FOXF2 limits mesenchymal Wnt signalling and promotes extracellular matrix production, particularly of collagens (24). It may have a similar role in the crosstalk between cell layers in the developing cornea. Early-onset glaucoma patients with a segmental duplication encompassing both FOXC1 and FOXF2 display increased corneal thickness, and a role for FOXF2 in this phenotype remains a possibility (25).

Our study also furthered our knowledge on the recently published CCT QTLs, upstream of ZNF469 and FOXO1. The association between CCT and the common variant rs2755237 upstream of ZNF469 replicates the finding reported by Lu et al. (13), with similar effect size estimates (0.21 SD for our study, 0.17 for the Lu et al. study). This suggests that the causative variant(s) is in high LD with this SNP in all of the diverse populations tested. It is likely to affect the long-range regulation of the downstream gene ZNF469, encoding a ZNF protein of unknown function in which rare mutations can cause BCS (5), but this remains to be proven experimentally. In our study, the effect of SNP rs2755237 upstream of FOXO1 was in the same direction but with a smaller effect size in all four Croatian/UK population samples compared with those reported by Lu et al. (13), in which this locus was genome-wide significant. This suggests that there is a real QTL at this locus which was not statistically significant in our study (meta-analysis P = 0.01) due to its more modest sample size. The fact that the MAF for the associated SNP is smaller than for the 16q24 variant (around 13% rather than 30%) also makes it more prone to sampling variation. Interestingly, a rare SNP (MAF <0.02), rs17592094, located 17.9 kb upstream of rs2755237, shows an association at P < 10^-5 in our combined Croatian/UK meta-analysis of imputed data. The effect size is very large in all the four populations tested, up to 1 SD (Supplementary Material, Table S1). The high LD between rs17592094 and rs2755237 (D′ = 1 in the CEU HAPMAP data set) suggests that there may be one or more rare variants of major effect in this region that may be tagged by the common SNP reported (23,26). Unlike other members of the Forkhead transcription
factor family, such as FOXC1 (25,27,28). FOXO1 has no documented role in the development of the ocular anterior segment but it is expressed in the cornea (29). FOXO1 has recently been shown to be one of the targets for transcriptional regulation by FOXC1, in which mutations are associated with various anterior segment malformations and glaucoma in Axenfeld-Rieger syndrome (30).

Finally, our study highlighted a new candidate QTL (rs10471977) on 5q11.2, which showed suggestive significance in the combined Croatian/UK series ($P = 3.7 \times 10^{-7}$) and had a stronger effect size than the QTL upstream of ZNF469, but with a lower MAF, making it less statistically significant. The effects of this QTL were noticeably stronger in the three Croatian population samples than in the UK or Australian population samples, suggesting a population-specific variant or a different LD pattern in the British-heritage samples. There was no biologically supported candidate gene in the vicinity of this QTL, although both flanking genes, ANKRD55 and MAP3K1, are thought to be involved in cellular signalling. Interestingly, there are two FOXO1 binding sites in the ANKRD55 gene promoter, suggesting that they could be acting in the same pathway (http://www.sabiosciences.com/chipqpcrsearch.php?species_id=0&nfactor=n&ngenene=n&B2=Search&src=genecard&factor=Over+200+TF&gene=ANKRD55) last accessed 22/08/2010.

GWAS promises to be a powerful tool to unravel the genetic architecture underlying CCT with already five genome-wide significant QTL associations identified and replicated in at least some populations, which should shed new light on the underlying biology and provide new targets for identifying genetic variation influencing corneal thickness both in anterior segment diseases and the response to refractive surgery.

**MATERIALS AND METHODS**

**Subjects**

The four populations (CROATIA-Vis, CROATIA-Korčula, CROATIA-Split, ORCADES) used for analysis comprise healthy adult volunteers from the Croatian islands of Vis and Korčula, the Croatian urban city of Split and the northern isles of Orkney (Orkney Complex Disease Study, ORCADES, Scotland, UK). The studies received approval from relevant ethics committees in Scotland and Croatia and followed the tenets of the Declaration of Helsinki. These populations have all participated in many other GWAS of medically relevant quantitative traits (31). Recruitment and ocular measurements performed in Vis and Korčula has been described in detail elsewhere (11). Briefly, the Vis study included unselected adult participants, aged 18–93 (mean $= 56$), a subset of which ($n = 640$) underwent a complete eye examination in summer 2007 and provided their ophthalmologic history. The Korčula study included a total of 969 examinees, aged 18–98 (mean $= 56.3$), and most ($n = 930$) underwent a complete eye examination. ORCADES is an ongoing family-based, cross-sectional study in the Scottish archipelago of Orkney. Among 1285 individuals with eye measurements, only 529 (aged 22–88 (mean $= 55.1$)) have been genotyped and passed genotyping quality control and were used in this analysis. The Split study is an ongoing cross-sectional population study in the Croatian city of Split; 499 individuals with whole genotype scans were
available, aged 18–85 (mean = 49.04), 372 of whom had undergone a complete eye examination.

Eye examination and measurements

CCT was recorded along with other ocular biometric measurements using a Nidek Echoscan US-1800 A-scan device in all three Croatian studies after application of sterile oxybuprocaine anaesthetic eye drops (Minims-Chauvin Pharmaceuticals Ltd). The Orkney measurements were performed using an IOPac ultrasound pachymeter (POD; Heidelberg Engineering). In the analyses, CT measures were transformed into Z-scores (individual trait value-population mean/population SD). Measures on eyes with a history of trauma, intra-ocular surgery or LASIK operations were removed. Right eye values were plotted against the left eye values. Pearson correlations for right and left eyes were highly statistically significant for CCT (two-tailed significance level of 0.01): 0.9 for Korčula and Vis, 0.92 for Split, 0.97 for ORCADES. Given the high correlations between right and left eye measures, the analysis was done on the right eye measures, unless the left eye had more complete measurements (e.g. due to trauma or cataract surgery on the right eye).

Genotyping and quality control

Genotypes were generated using a dense Illumina SNP array, HumanHap 300v1 for Vis, a mix of HumanHap 300v2 and 370CNV-Quad for ORCADES, 370CNV-Quad for Korčula and 370CNV-Quadv3 for Split, following the manufacturer’s standard recommendations. Genotypes were determined using the Illumina BeadStudio software. Samples with a call rate below 97% [for SNPs with call rates above 98%, a MAF above 2% and a P-value for Fisher’s exact test of Hardy–Weinberg equilibrium (HWE) above 10^{-4}], potentially mixed samples with excess autosomal heterozygosity or gender discrepancy (based on the sex chromosomes genotypes) and ethnic outliers (based on principal components ancestry principal components were obtained following multidimensional scaling of identity by state distances using the ibs and mds functions in GenABEL).

- **Quality control**: After this quality control step, the package GenABEL (32). Measures on eyes with a history of trauma, intra-ocular surgery or LASIK operations were removed. Right eye values were plotted against the left eye values. Pearson correlations for right and left eyes were highly statistically significant for CCT (two-tailed significance level of 0.01): 0.9 for Korčula and Vis, 0.92 for Split, 0.97 for ORCADES. Given the high correlations between right and left eye measures, the analysis was done on the right eye measures, unless the left eye had more complete measurements (e.g. due to trauma or cataract surgery on the right eye).

- **Statistical analysis**: Z-scores were used for the association analysis (calculated by adjusting CCT measures for age and the three first principal components of ancestry and standardizing residuals using the ztransform command in GenABEL) to allow comparison of effect size across samples and for the meta-analysis. The ancestry principal components were obtained following multidimensional scaling of identity by state distances using the ibs and mds functions in GenABEL.

- **Genome-wide association analysis**: Genome-wide association analysis was performed using GenABEL [or the probABEL package (33) for imputed data] using an additive SNP allelic effect model and correcting for family relatedness using the polygenic (34) and mmscore (35) functions implemented in GenABEL. The results from all cohorts were combined into a fixed-effects, additive model meta-analysis using inverse variance weighting implemented in MetABEL (http://mga.bionet.nsc.ru/~yurii/ABEL/). The threshold for genome-wide significance was set to 5 × 10^{-8} for the meta-analysis of imputed data (36).

- **Forest plots for meta-analysis**: Forest plots for meta-analysis of individual SNPs and heterogeneity measures were obtained using the meta package in R (http://cran.r-project.org/web/packages/meta/index.html).

- **The metric used for between-population heterogeneity**: The metric used for between-population heterogeneity was I^2. LD blocks were defined by SNPs from NCBI releases 21, 22 and HapMap3_r3 with the LD measure r^2 ≥ 0.8 for the associated SNP.

- **Plots of the P-values against genomic position**: Plots of the P-values against genomic position were made using Haploview (37) and SNAP (38). LD between associated SNPs and SNP from releases 21, 22 and HapMap3_r2 were performed using SNAP. URLS: http://mga.bionet.nsc.ru/~yurii/ABEL/, http://www.broadinstitute.org/haploview/, http://www.broadinstitute.org/mpg/snap/ and http://cran.r-project.org/web/packages/meta/index.html.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank Biljana Andrijević Derk, Valentina Lacmanović Lončar, Krešimir Mandić, Antonija Mandić, Ivan Škergo, Jasna Pavičić Astaloš, Ivana Merc, Miljenka Martinović, Petra Kralj, Tamara Knežević and Katja Barač-Juretić from diverse university and hospital ophthalmology departments in Croatia for their participation in the Croatian field work. We would like to acknowledge the invaluable contributions of Lorraine Anderson, the research nurses in Orkney, in particular Margaret Pratt who performed the eye measurements, the administrative team in Edinburgh and the people of Orkney.

We acknowledge the Wellcome Trust Clinical facility (Edinburgh) for DNA extraction for the ORCADES study and genotyping the CROATIA-Vis study and Peter Lichner and the Helmholtz Zentrum Munchen genotyping staff (Munich, Germany) for genotyping the ORCADES and Korčula cohort.

Conflict of Interest statement. None declared.
FUNDING

This research was funded by grants from the Medical Research Council (UK) to A.W. and N.D.H., from the Republic of Croatia Ministry of Science, Education and Sports to I.R. (108-1080315-0302), from the Chief Scientist Office of the Scottish Government to J.F.W. (CZB/4/276, CZB/4/438, CZB/4/710), the Royal Society to J.F.W. and the European Union framework program 6 EUROSPAN project to H.C. (contract no. LSHG-CT-2006-018947).

REFERENCES