Twins Eye Study in Tasmania (TEST): Rationale and Methodology to Recruit and Examine Twins

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Visual impairment is a leading cause of morbidity and poor quality of life in our community. Unravelling the mechanisms underpinning important blinding diseases could allow preventative or curative steps to be implemented. Twin siblings provide a unique opportunity in biology to discover genes associated with numerous eye diseases and ocular biometry. Twins are particularly useful for quantitative trait analysis through genome-wide association and linkage studies. Although many studies involving twins rely on twin registries, we present our approach to the Twins Eye Study in Tasmania to provide insight into possible recruitment strategies, expected participation rates and potential examination strategies that can be considered by other researchers for similar studies. Five separate avenues for cohort recruitment were adopted: (1) piggy-backing existing studies where twins had been recruited, (2) utilizing the national twin registry, (3) word-of-mouth and local media publicity, (4) directly approaching schools, and finally (5) collaborating with other research groups studying twins.

Keywords: ophthalmology, glaucoma, myopia, genetics, genome-wide association

Toward an Understanding of Inherited Eye Disease

Visual impairment is a leading cause for morbidity and poor quality of life in our community. Unravelling the mechanisms underpinning important blinding diseases could allow for preventative or curative steps to be implemented (Bainbridge et al., 2008; Maguire et al., 2008). The dramatic increase in our general understanding in genetics over the previous 50 years has been particularly revealing for many eye diseases. There are many Mendelian genetic eye diseases for which genes have been identified (MacDonald et al., 2004); however, the search for genes underlying complex genetic traits has met variable success.

Of the five leading causes of blindness in developed countries, only age-related macular degeneration (AMD) has been found to have a major gene effect, at the *CFH* locus (OMIM:610698), identified through a genome-wide association study (GWAS) (Klein et al., 2005). For the remaining four diseases — glaucoma, cataract, refractive error and diabetic retinopathy — there has been limited success in uncovering associated genes through the study of large families. In glaucoma the *MYOC* gene (OMIM:137750) has been found to account for approximately 3% of unselected open-angle glaucoma cases with firm clinically important genotype-phenotype correlations (Hewitt et al., 2006). Mutations in genes leading to other rare glaucoma syndromes have also been identified: *OPTN, CYP1B1, PITX2, FOXC1* and *PAX6* (Mackey, 2008). One large GWAS identified *LOXL1* (OMIM:177650) as a contributory factor for exfoliation syndrome, a major risk for glaucoma (Hewitt et al., 2008; Thorleifsson et al., 2007). Recent work has uncovered a significant association between variants in the *EPHA2* gene (OMIM:176946) and age-related cataract (Jun et al., 2009; Shiels et al., 2008; Zhang et al., 2009). Along with the *EPHA2* gene, many others have been found to cause congenital or syndromic cataract (MacDonald et al., 2004). Despite the identification of multiple putative loci associated with nonsyndromic myopia, no causative gene has yet been found (Hornbeak & Young, 2009). Similarly, no major genetic associations

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predisposing to diabetic retinopathy have been identified (Abhary et al., in press).

The Glaucoma Inheritance Study in Tasmania and the Need for a Twin Study

Our work with the Glaucoma Inheritance Study in Tasmania (GIST) identified several bottlenecks precluding the identification of glaucoma-causing genes, notably an inconsistent definition of the disease (Coote et al., 1996). The three historic pillars of glaucoma diagnosis include: an elevated intra-ocular pressure (IOP), optic disc cupping and corresponding visual field loss. Despite attempts to standardize diagnosis, accurate case definition remains a major problem, particularly due to difficulties in phenocopy, such as that occurring with ocular hypertension and normal tension glaucoma. The late age of onset (usually after 40 years of age) and the fact that approximately half of all glaucoma cases in our community are undiagnosed, means large pedigrees are difficult to locate and study. Additionally, many pedigrees overlap and molecular studies suggest that many different susceptibility genes are likely to interact (Sack et al., 1996; Vincent et al., 2002).

To overcome these issues, we decided to break glaucoma down into its component measures or endophenotypes, with the aim of investigating these as quantitative traits (QT). Although glaucoma is often associated with an elevated IOP and excavated optic cups, we questioned whether it would be possible to identify genes that affect these measures in normal people and then to investigate these as candidate genes for glaucoma. The Brisbane Adolescent Twin Study (BATS; Wright & Martin, 2004), which identified the genes associated with eye color in a predominantly Caucasian population and found several genes associated with albinism, exemplifies this approach (Zhu et al., 2004). Despite none of the twins in the BATS study having albinism, the discrete classification of participant's eye color (into brown, hazel/green, or blue) was sufficient to identify the polymorphisms in the genes that cause variable eye color, while severe mutations in the same genes have been shown by others to cause albinism (Zhu et al., 2004). The aim of this paper is to outline the study design and recruitment methodology for our Twins Eye Study. The QTs that we specifically targeted included the component measures of glaucoma; IOP and cup-to-disc ratio and the confounding measures; central corneal thickness, optic disc size and refraction.

Classical Twin Design and Other Twin Eye Studies

Twin siblings provide an excellent natural experiment in which to investigate important genetic QTs. One can conduct a classic twin study comparing the correlation and covariance of phenotypes within monozygotic (MZ) twin pairs with that within dizygotic (DZ) twin pairs to estimate the heritability of a trait.

While population-based and case-control studies can be used for genome-wide association studies (GWAS), twins can also be used for GWAS, with both siblings from DZ twins pairs and one of each MZ pair; there is only a slight loss of power for GWAS from examining twins rather than unrelated individuals (Visscher et al., 2008). In addition to GWAS, data from twin siblings can also be used for linkage and within-pair analysis, including the discordant MZ twin pairs to look for environmental and epigenetic factors.

The first classic twin study examined the contribution of heredity factors to refraction in human eyes (Jablonski, 1922). There have been several twin studies of glaucoma and related traits in the literature but most of these are small, had limited power and did not use modern statistical analysis method such as structural equation modeling. These included investigation into the heritability of steroid responsiveness, (Schwartz et al., 1972, 1973a, 1973b, 1973c) physiological size of the optic cup, (Schwartz et al., 1975, 1976; Teikari & Airaksinen, 1992) and glaucoma itself (Gottfredsdottir et al., 1999; Teikari, 1987, 1988, 1990; Teikari et al., 1987a; Teikari et al., 1987b). Other concurrent twin eye studies in Australia are investigating AMD and early phenotypes in individuals over age 50 years (Chamberlain et al., 2006) and myopia and refractive error in individuals aged 18–50 years (Dirani et al., 2006).

Study Design and Methodology

Twins Eye Study in Tasmania Recruitment

The twins we examined in the Twins Eye Study Tasmania (TEST) were recruited in several waves, many overlapping with each other. Ethical approval was obtained from the Royal Victorian Eye and Ear Hospital, the University of Tasmania, the Australian Twin Registry (ATR) and the Queensland Institute of Medical Research (QIMR). The estimated number of twins born in key years is shown in Table 1. We adopted five different methods of identifying twins for participation in a total of seven overlapping waves of recruitment:

A. Piggy-Back Existing Study Where Twins Had Been Recruited

1. Commencing in late 2000, twins who had been involved in the Menzies Centre for Population Health Research (now Menzies Research Institute) Tasmanian Infant Health Study (TIHS) were recruited as our initial cohort. This birth cohort was originally recruited between 1988 and 1992 to investigate the etiology of sudden infant death syndrome (SIDS) (Dwyer & Ponsonby, 1992). The twins had been recently examined at ages 9, 10, and 11 years for a fitness study (Dwyer et al., 1999). We were able to correlate early birth data with later ocular biometry (Ponsonby et al., 2007). This recruited 326 individuals. However, other cohort recruitment listed below increased the total number of TIHS participants in the TEST to 346 individuals (172 multiple birth sets) (Ponsonby et al., 2007). Because it is the parents who decide whether children participate in a study, less skewing

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Table 1

Births per Year in Tasmania During Selected Years of Interest (Australian Bureau of Statistics)

Year	Total number of recorded births	Estimated number of twin pairs*	Number of complete twin pairs recruited
1961	8982	157	13
1988	6779	119	75
1989	6813	119	56
1990	7043	123	52
1991	6870	120	82
1992	6987	122	34

Note: * Calculations assume a twin birth rate of 3.5% of all live births (Australian Twin Registry).

by gender and zygosity is seen compared with studies involving adult twins.

2. The twins in the Tasmanian Asthma Study (TAS) were also directly approached. This was the follow-up of Tasmanians born in 1961 who had been examined in 1968 and followed for respiratory problems (Wharton et al., 2006). As this cohort was to be examined further and genetic studies had been proposed, we arranged to help locate all the previously seen twins. Of 91 twin pairs in the TAS study, eight had already participated and 15 had withdrawn or were deceased. This cohort of middle-aged twins was quite problematic to enroll. Many who agreed to participate had been in the ATR cohort. Of those new participants, when both twins agreed to participate, it was often difficult to arrange a visit and in many cases only one twin was examined. In total, 23 new people (from 42), nine pairs and five halves were examined. This brings the numbers examined to 23/136 (16.9%) or 39/152 (25.7%) if we included the 16 people previously recruited.

B. Utilize the National Twin Registry

3. The second major wave of recruitment was conducted through the ATR (http://www.twins.org.au). We asked all twins aged 5 years and older to participate. In addition to adding to the adolescent twins, we enrolled twins of all ages to allow estimation of age effects on ocular biometry across the cohort. The Tasmanian enrolment in the ATR is on par with the national average given that Tasmania has 2.5% of the national population. (Australian Twin Registry) The number of twins in the ATR who agreed to participate is shown in Table 2. This is the number of twins who agreed to participate and not necessarily the number who did complete the eye examination. This arm of the study needed to convince both twins to participate. However, in minors it was usually the mother who decided that it was a good idea to be involved and thus none of the twins aged 5 to 17 years had only one twin agreeing to participate. As with the registry itself and most adult twin registries, there was a skew of female twins and MZ twins on the registry and agreeing to participate. The response rates were comparable to other twin studies facilitated by the ATR (Table 3) and almost the same as another eye study of AMD in Victoria (Chamberlain et al., 2006).
4. The ATR was also used to recruit twins aged 17 and 18 years resident in Melbourne who were too young to participate in the earlier AMD (Chamberlain et al., 2006) and myopia studies (Dirani et al., 2006).

Table 2

Number of Australian Twin Registry Participants Who Agreed to Participate in the TEST

Gender	Age group (years)	Zygosity	Total Pairs Approached	Yes	No	Only 1 Yes	No Reply	% both agreed to participate
F/F	5–17	DZ	29	11	8	0	10	38
F/F	5–17	MZ	38	17	6	0	15	45
F/F	5–17	?Z	9	4	1	0	4	44
M/F	5–17	DZ	73	26	21	0	26	36
M/M	5–17	DZ	35	10	13	0	12	29
M/M	5–17	MZ	34	11	12	0	11	32
M/M	5–17	?Z	10	3	1	0	6	30
F/F	18–80	DZ	102	27	33	8	34	26
F/F	18–80	MZ	135	49	42	7	37	36
M/F	18–80	DZ	114	19	20	18	57	17
M/M	18–80	DZ	83	8	19	8	48	10
M/M	18–80	MZ	67	14	12	5	36	21
Externally recruited twins who were as listed on the ATR:								
—	—	—	40	37	3	0	0	—
Total pairs listed on the ATR:			686	228	172	38	248	33

Note: Abbreviations: F/F: Female/Female; M/M: Male/Male; M/F: Male/ Female; DZ: dizygotic pairs; MZ: monozygotic twin pairs; ?Z: unknown zygosity; ATR: Australian Twin Registry.

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Table 3

Participation Rates in Other Twins Studies Conducted Through the ATR (www.twins.org.au).

Study topic	Entry Criteria	% Positive response rate (both 'yes')
Breast density	F/F, 40–70yrs	60
Age-related macular degeneration	F/F, M/M, M/F, > 50	35
Baldness, dandruff and greying	F/F, 18–75yrs	45
Genetics of reading ability	F/F, M/M, 3.75–5yrs	62
Smoking and bone density	F/F, M/M, 40–85yrs	3
Tooth emergence	F/F, M/M, M/F, 0–1yr	60
Behavior	F/F, M/M, M/F, 5.75–17yrs	62
Epilepsy	F/F, M/M, 18–40yrs	10
Borderline personality disorder	F/F, M/M, M/F, 18–32yrs	14
Psychosis study	F/F, M/M, M/F, 18–65yrs	6

Note: Abbreviations: F/F: Female/Female; M/M: Male/Male; M/F: Male/ Female.

The ATR sent letters to 458 sets of twins. This recruited 128 individuals (Table 4); however, we did not pursue these twins extensively to participate and many were not available at the time of assessment because of secondary school exams.

C. Word-of-Mouth and Local Media Publicity

5. The third major method of recruitment in Tasmania was by word of mouth. This included twins who became aware of the study through media publicity, through parents in a twin support organization or twins seen as family members in the GIST. Although this involved some potential ascertainment bias, the rate of a positive family history of a first-degree relative with glaucoma in the adult twins was 11.3% (Green et al., 2007). In addition four sets of twins in the Norfolk Island Eye Study agreed to be in the TEST.

D. Approach Schools

6. The 'Tasmanian Schools Approach' was a mail-out through the high schools in Tasmania. This method had been used successfully to establish the BATS Cohort (Zhu et al., 2003). We contacted the education department and obtained approval from the Minister and Director and then wrote to the headmasters of every public and private school asking them to participate. Participating schools identified twins attending and forwarded an invitational letter to parents who then contacted our group. Prior to the mail-out we had seen about 210 pairs of twins in Tasmania aged < 18 years. Of 104 schools contacted, 84 agreed to participate, two declined and 18 did not respond. Of those schools that agreed, 13 had no twins to report. Schools that did not respond were mailed once, phoned twice and emailed once. In total, 326 invitation letter packs were sent to 71 schools. One pack per multiple birth-set was sent to the school to give to the family. Forty-seven letter packs were completed and returned. Eight of the 47 had already been seen by the TEST. Others who did not respond may have already participated. Forty-four multiple birth sets (including two sets of triplets and two families with two sets of twins) agreed to participate while three declined. Of these, 21 new sets of twins and one set of triplets were actually examined by the study. Although 24/326 multiple birth sets would seem a low recruitment, we do not know how many of the 326 had actually already been seen.

Table 4

Number of 17- and 18-Year-Old People Listed on the Victorian Australian Twin Registry

Criteria	No of twins listed
F/F; DZ/Unknown, 17–18 yrs, both living in Victoria	124
M/F; DZ, 17–18 yrs, both living in Victoria	183
M/M; DZ/Unknown,17–18 yrs, both living in Victoria	151
Total	458

Note: Abbreviations: F/F: Female/Female; M/M: Male/Male; M/F: Male/ Female; DZ: dizygotic pairs.

E. Collaborate With Other Research Groups Studying Twins

7. The final wave, in which twins were examined concurrently with the above approaches, was recruited from the BATS at QIMR. These twins were initially recruited from the age of 12 years as part of a skin cancer genetic study 'the Mole Study'. (Zhu et al., 2004) Most participants had been involved in four previous twin studies where they had been examined. We initially targeted DZ twins but later included all MZ twins, commencing with the older cohort moving down from twins born in 1978 to those born in 1994 over a 4-year examination window. The number of BATS participants who agreed to be in the Eye study is shown in Table 5. In addition 196 siblings of twins also participated in the eye examination. This provided a cohort of singleton births to compare with the data from the twins to investigate if there were any differences between singleton and twin participants in relation to ocular biometry. If there were no differences, then we could conclude that twins were representative of the Australian general population. Some ATR twins from around Australia, including Tasmania, were included in QIMR-related projects for DNA analysis. The other major advantage this cohort gave to the study was the possibility of investigating latitudinal variation in disease, much of which is due to differing levels of sunlight, particularly UV exposure. It is firmly established that UV exposure contributes to several diseases affecting the eye, e.g. pterygium and cortical cataract.

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Table 5

Brisbane Adolescent Twins Study Participation Rate in the Twins Eye Study

Criteria	Total individuals approached	Number participated	Participation rate (%)
F/F; DZ	293	153	52
F/F; MZ	432	229	53
F/F; ?Z	2	2	100
M/F; DZ	569	288	51
M/M;DZ	299	151	51
M/M;MZ	369	155	42
M/M; ?Z	0	0	–
Siblings M	234	98	42
Siblings F	245	123	50
Total	2443	1199	49

Note: Abbreviations: F/F: Female/Female; M/M: Male/Male; M/F: Male/ Female; DZ: dizygotic pairs; MZ: monozygotic twin pairs; ?Z: Unknown Zygosity.

TEST Ophthalmic Examination

As we wished to utilize ophthalmic instrumentation that was difficult to access due to associated expense and limited availability, we used private or hospital ophthalmology clinics out of hours (weekends and early evenings). Twins were requested to attend together, which occurred for all children and the majority of adults. If it was not possible for a twin pair to attend on the same day, we endeavoured to see them at the same time on different days, to standardize diurnal variation in factors such as IOP (although this proved to be a challenge with one pair of MZ twins who were nurses working different night and day shifts). In several cases we were only able to examine the first of the twin pair as the second twin resided in another state. Field trips were conducted in every Australian capital city in an attempt to reconcile these incomplete paired examinations.

The initial examination protocol for young subjects (< 15 years) involved a 91-item questionnaire from which each child's demographic and ocular history data were drawn and this was completed by parents at home or in the clinic. Consent paperwork was also completed by the parents and children underwent an ocular examination of one hour duration.

During the examination, a researcher would check that the forms were completed and assist in explaining any queries. A small award ceremony involving a chocolate medal for the 'Eye Olympics' occurred prior to fundus photography. As more equipment, such as the pachymeter and the IOL Master, became available, participants were invited back to complete these tests. In adults, blood or Oragene saliva samples were collected for DNA testing (zygosity confirmation and genome-wide scans). In children, buccal swabs or Oragene saliva samples were collected. In adolescents, or when repeat examination was conducted several years later, a blood sample was taken and those participants who were now adults signed their own consent.

Our full examination protocol is detailed in the appendix and consisted of:

1. Visual acuity
2. Binocular vision function tests
   (a) Cover test
   (b) Four dioptre base-out prism test
   (c) Ocular motility
   (d) Stereopsis (Lang/Titmus)
3. Color vision (Ishihara)
4. Eye dominance
5. Conjunctival UV auto-fluorescence photos
6. Refraction (pre and post-cycloplegic autorefraction)
7. IOP (Tonopen or Goldmann)
8. Ultrasound pachymetry
9. A-scan or IOL Master
10. Dilated stereoscopic optic disc photographs (Nidek 3-DX)

The examination was conducted by a team consisting of an ophthalmologist and an orthoptist, with additional staff including ophthalmology trainees, medical students, optometrists and medical photographers depending on the number of twins to be seen. The clinical experience of the field researchers allowed explanation and trouble-shooting problems when abnormalities were detected.

Inclusion and Exclusion Criteria

Informed written consent was obtained from at least one parent of each child, coupled with verbal assent from all children. We did not adopt any exclusion criteria at the time of examination. We included individuals with a family history of any eye disease, and even genetic anomalies such as Trisomy 21 and Klinefelter's syndrome (XXY). Table 6 displays the eye pathologies identified during this study.

Total Participant Numbers and Associated Costs

The total number of twin pairs are listed in Tables 7. In addition, there were 55 triplet individuals (19 sets of triplets fully attended and two sets with only two of the triplets participating), 87 single twins, several families with second sets of twins (seven twin pairs and one single twin participated), and 196 siblings participated. Figure 1 illustrates the age breakdown of the twin and sibling participants. The overall cost for each fully phenotyped person with microsatellite zygosity testing were approximately AUS$700. This study examined 2,235 people at total cost of approximately AUS$1,564,500.

Triplets and Multiple Sets of Twins in Families

When coding participants it is important to develop number coding systems for twin individuals and families. These can be problematic when there are triplets or when there are two sets of twins within the same family (either parents or siblings). These extra areas of relatedness need to be recognized for incorporation into future analysis (though will become apparent on

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Table 6

Number and Concordance of Twins Pairs with Previous Ocular Disease

Disease	Number of cases in the TEST	Number of cases in the BATS	Number of affected concordant twin pairs (MZ/DZ)	Number of affected disconcordant twin pairs (MZ/DZ)
POAG	12	0	4/1	1/1
ROP	6	0	2/1	0/0
Congenital cataract	4	0	0/2	0/0
Keratoconus	2	1	0/0	1/2
Corneal dystrophy	2	1	0/0	0/3
Congenital glaucoma	1	1	0/0	1/1
Congenital Horner's syndrome	2	1	0/0	1/2
RVO	1	0	0/0	1/0
AION	1	0	0/0	1/0
Ocular albinism	0	2	1/0	0/0
LHON	0	1	0/0	0/1
RP	0	1	0/0	0/1
Anterior uveitis	0	1	0/0	1/0
Penetrating eye injury	0	1	0/0	0/1

Note: Abbreviations: TEST: Twins Eye Study in Tasmania; BATS: Brisbane Adolescent Twins Study; MZ: monozygotic twin pairs; DZ: dizygotic pairs; POAG: primary open angle glaucoma; ROP: retinopathy of prematurity; RVO: retinal vein occlusion; AION: anterior ischaemic optic neuropathy; LHON: Leber's hereditary optic neuropathy; RP: retinitis pigmentosa

initial GWAS cleaning). In our study we examined seven pairs of twins and one single twin where this occurred. In total 55 triples (19 sets of triplets, where two sets had only two siblings attended) and 196 siblings were recruited. An additional 87 unrelated people were recruited where their corresponding twin sibling did not participate.

Births in Victoria for the year 2003 documented 1106 sets of twins, representing 3.5% of all births, 18 sets of triplets, representing 0.1% of all births and one set of quads, representing less than 0.1% of all births (Australian Twin Registry). For the year 2004 there were 1,123 sets of twins, representing 3.5% of all births and 16 sets of triplets, representing 0.1% of all births. These numbers will differ according to location and time, with factors such as ethnicity and practices in reproductive technology having an impact. In the TEST we examined 19 sets of triplets: five from Tasmania, 13 from Brisbane and one from Victoria. This included trizygotic (all three different), DZ (i.e., a MZ pair and a sibling) and MZ (all three identical) triplets. DZ triplets would ultimately be included in the twin study where the comparison of MZ and DZ twins is internally controlled within the birth cohort.

Table 7

Total Number of Twin Pairs Recruited in the Study, by Zygosity and Study

Zygosity	TEST	BATS	Total
F/F; MZ	132	111	243
M/M; MZ	68	74	142
F/F; DZ	87	73	160
M/M; DZ	69	71	140
M/F; DZ	131	134	265
Total	487	463	950

Note: Abbreviations: F/F: Female/Female: M/M: Male/Male: M/F: Male/ Female: DZ: dizygotic pairs; MZ: monozygotic twin pairs.

Collaborating Replication and Extrapolation Samples

Replicate data are important in a scientific study to ensure reliability and validity. In addition to our work, two other international groups are collecting multiple biometry measurements in large-scale twin eye studies. The first cohort, the TwinsUK Eye Study from St Thomas' Hospital in London, is coordinated by Dr Christopher Hammond. The majority of these twins are older than 50 years and the cohort is predominantly female. The TwinsUK group has performed several ocular heritability studies and its myopia study involved a genome-wide microsatellite linkage analysis on 221 DZ twin pairs with 400 STR markers using non-parametric multipoint linkage techniques. Analyses discovered linkage at chromosomes 11p13 (LOD 6.1) where the *PAX6* is located, as well as 3q26 (LOD 3.7), 4q12 (LOD 3.3), and 8p23 (LOD 3.3) (Hammond et al., 2004).

The Guangzhou Twin Eye Project involved recruiting 1,000 sets of twins aged seven to 15 years living in the proximity of the Zhongshan Eye Hospital in Guangzhou, China (He et al., 2006). The main phenotype of interest was myopia. As these twins were examined annually for five years (usually over a 7-week period in their summer school holidays), additional phenotypic measures were added at subsequent visits. This allowed the researchers to borrow

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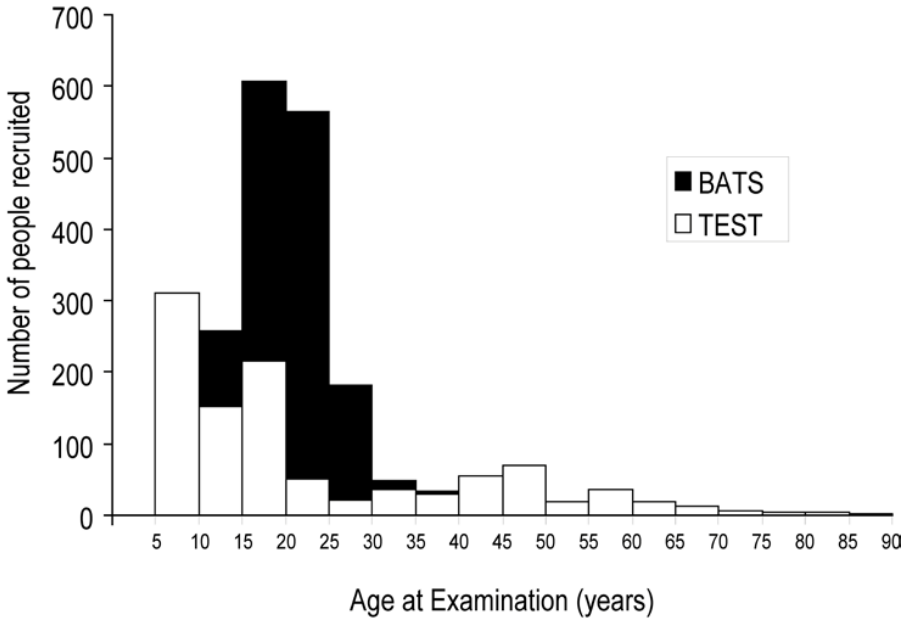

Figure 1
Age distribution of participants recruited.

expensive equipment for a short period of time. Although this study has the most comprehensive ocular phenotyping of those discussed, there are currently no adult twins involved and no DNA analysis has been funded (He et al., 2008a; He et al., 2008b). Prior to commencing the Guangzhou project, the Chinese team (directed by Dr Mingguang He) travelled to Australia in order to discuss and develop efficient screening strategies for their study cohort. The group subsequently examined 700 twin pairs in seven weeks with 'production line' efficiency. In the second year of examination they utilized the Visante Anterior Segment optical coherence tomography (OCT) to evaluate anterior chamber angle biometry as a QT (He et al., 2008a; He et al., 2008b). Several anterior chamber (AC) parameters were examined and high heritabilities estimated for each trait: angle opening distance (AOD), angle recess area (ARA) and trabecular–iris space area (TISA). These AC measurements, in conjunction with the AC depth measured with the IOL Master (Carl Zeiss, Jena, Germany), may give clues to the genetic risk factors for angle closure glaucoma. China's one child policy has made family genetic research in China a challenge but has made identification of twins much easier.

The various studies have learnt extensively from each others' experiences. The other Australian twins in the AMD and myopia studies have not undergone glaucoma-associated endophenotyping and no genome-wide analysis has been funded to date (Chamberlain et al., 2006; Dirani et al., 2006).

Instructive Issues Identified During the Study

Some children placed on the ATR by their parents were not keen to participate. We explained to them that they were never obliged to participate and that their status could be recorded as non-active. Some children declined parts of the examination — for example, administration of drops. It was a challenge to respect both the child's autonomy and the parents' authority, but usually we were able to come to an acceptable compromise in the data collection.

Results: Preliminary Summary

Preliminary analysis of pachymetry suggested a high heritability, so we combined the available Australian data with the UK data to analyze heritability using the Mx program (Neale et al., 2002) and found a 95% heritability (Toh et al., 2005b). We also looked at a new approach to identify optic disc parameters that would be heritable (Hewitt et al., 2007). Identical twins eyes are very similar, but can always be distinguished by iris recognition-screening software. Similarly the retinal vessels and optic nerve are similar in identical twins but can be distinguished (Daugman & Downing, 2001). With respect to optic disc photographs, clinicians can pick MZ from DZ twins with 80–90% accuracy (Hewitt et al., 2007). We used existing microsatellite data to analyze linkage for axial length (AL) using 1790 eyes (Zhu et al., 2008). There was a kurtosis to the normal distribution. There was higher correlation for MZ compared with DZ twins, giving additive genetic effect of 81% with further 11% individual and 8% eye-specific environmental effects. Using 796 short tandem repeat microsatellite markers, we found several areas of interest. Two were near previously identified myopia loci on chromosomes 4 and 12, but another locus on chromosome 5 was the most significant. This overlapped the region

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for Wagner syndrome and its associated gene *Versican* (*chondroitin sulfate proteoglycan 2;* OMIM no. 143200) a principal constituent of the extracellular matrix with a key role in tissue morphogenesis (Wight, 2002) participating in cell adhesion, proliferation and migration and expressed in the sclera. Splice variants have been implicated in Wagner disease, a dominantly inherited vitreoretinopathy, associated with mild to moderate myopia. We also used the existing microsatellite marker data to perform linkage analysis on retinal vessel measurements (Sun et al., in press).

Conclusions

The aim of this article is to provide a practical guide to researchers commencing a twin eye study. We have demonstrated that although a twin registry is helpful, it is not essential to recruit child and adolescent twins and that a school approach is useful for targeting this age group. Participation rates are less than 50% for all recruitment methods. Recruiting younger twins helps balance gender and zygosity. Working adults are the most difficult to recruit. A production-line examination protocol allows the most efficient examination. Maximum research return comes from the combination of studies so that instead of just an additive effect, there is crossover value of the studies. Twins understand genetics and are happy to be in multiple studies. Although GWAS can be done on unrelated individuals, twins allow linkage analysis as well and there is minimal loss of power with associations with twins of unrelated individuals (Visscher et al., 2008). Further information about our twins eye study is available online at www.twinseyestudy.com.

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Appendix A: Examination Protocol

Pre-Examination

Prior to a scheduled examination session, a member of the research team would ensure that the administrative components of the protocol were appropriately addressed. An appointment list of attending participants was reviewed and personal information and study family numbers (if assigned) checked for accuracy. Information about special requests and tests was highlighted on the appointment list, particularly if the subject had previously attended. The day before the examination, an SMS text message was sent to each participant as a reminder of his/her appointment time. Examination rooms were allocated a test name and room number to minimize participant confusion and optimize flow ('Have you been in room 5 yet?'). Study numbers were generally allocated at the time of participant presentation and cross-referenced with previous study numbers. Questionnaire and consent paperwork proformas were organized on clip-boards with attached pens and ID badges prepared for the younger twins.

On arrival, participants were welcomed and thanked and then received an explanation of the study and had any questions answered. Paperwork was provided (if not already completed) and parents allowed time to complete the forms (history, family history, consent). Although a routine order of tests was possible with multiple family members needing to be examined, pre-dilation examinations were done in different order to speed up examination of the family. Prior to instillation of eye drops and at the end of the examination, it was checked that all tests had been completed.

Visual Acuity

Visual Acuity (VA) was measured in both eyes monocularly at a distance of 3 metres with a LogMAR Bailey-Lovie letter chart. If the participant wore spectacles or contact lenses, VA was tested with these and re-assessed with a pin-hole if 6/6 could not be achieved. Young participants were encouraged verbally by the examiner to read as far as they could, with the additional implication that the exercise was a 'competition' with the sibling. If the examiner suspected that letters were memorized, the participant was asked to read a specific line in reverse order. VA was recorded either in Snellen form by adding/subtracting correct/incorrect responses from the lowest line read or in logMAR form (6/6 = 0.0). VA information returned to the participant pertaining to their examination was provided in Snellen form and often explained in relation to 20/20 which, surprisingly, is more familiar to most people in metric Australia!

Binocular Vision Function

Cover Test. To evaluate ocular alignment in the primary position of gaze, a cover test was performed at both far (6 m) and near (1/3 m) distances. Corrective lenses were worn as for VA measurement; however, the cover test was repeated without correction if a history of strabismus or amblyopia was reported.

Four Dioptre Base Out Prism Test. This test is used to ascertain if a micro-strabismus and associated suppression area is present. Participants were asked to fix on a small, distance target with corrective lenses worn as for VA measurement. A 4 dioptre base out prism was introduced over one eye and the participant given the opportunity to make a fixation movement to fuse the images. This technique was then applied to the other eye. A negative response, that is, presence of a central suppression response was recorded if a compensatory eye movement to overcome the prism and restore binocular single vision was not observed.

Ocular Motility. Eye movements were examined using a pen torch, with participants instructed to keep their head stationary and follow the light with their eyes. Each of the diagnostic directions of gaze was assessed by moving the pen torch in a 'Union Jack' formation and any abnormalities recorded.

Stereopsis. The Lang I Stereo test provides a measure of stereoacuity or depth perception ability. Corrective lenses were worn as for VA measurement and the target with the finest spatial resolution that the participant could discern, recorded.

Color Vision

Color vision was tested in a subset of children and adolescents binocularly using Ishihara color plates.

Eye dominance. Eye dominance was measured using the Miles test, a method whereby the participant brings both hands together at arms length to create a small opening through which a distance object is viewed. The examiner notes by observation which eye the participant is using or by asking the participant to alternately close the right and left eye and report which eye still sees the target (Miles, 1930).

Conjunctival UV Auto-Fluorescence

Conjunctival UV auto-fluorescence photos were taken using the camera system developed by Coroneo and colleagues (Ooi et al., 2006; Ooi et al., 2007). Photographs were taken using both reflected visible light (control) and UV-induced fluorescence with the aid of two portable photographic systems. Each consisted of a height adjustable table equipped with subject head-rest, camera positioning assembly, digital single-lens reflex camera, macro lens, and filtered electronic flash. Each eye was photographed at 0.94 magnification, with separate views of the nasal and temporal regions of both eyes. Colored low-voltage light emitting diodes (LED) were positioned on stands in the subject's visual field, at approximately 35

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degrees to the camera–subject axis to aid fixation. The UV-induced fluorescence photography was based on standard principles, using a specially adapted electronic flash system fitted with UV-transmission filters (transmittance range 300 to 400 nm, peak 365 nm) as the excitation source. Subject fluorescence was recorded with a Nikon D100 (Nikon, Melville, New York, USA) digital camera and 105 mm f/2.8 Micro Nikkor (Nikkor, Melville, New York, USA) lens fitted with infrared and UV barrier filters. Thus, only fluorescence was recorded by the camera. Images were saved in RGB format at the D100 settings of JPEG Fine (1:4 compression) and large resolution (3,000 2,000 pixels). Some unwanted red light allowed by the UV transmission filter was eliminated by removal of the red channel in Adobe PhotoShop (Adobe Systems Inc, San Jose, California, USA), equivalent to the use of a cyan filter on the camera lens. Each photograph could be verified immediately after it was taken and reshot, if necessary, to obtain a better result. Pictures could be identified by data imprinting or, in our case, by sound recording (Ooi et al., 2006). In addition photos of eye colour were taken with a Nikon Coolpix 995 digital camera (Nikon, Toyko).

Refraction (Pre- and Post-Cycloplegia)

Pre-cycloplegic refractive errors for both eyes were measured using a Humphrey-598 automatic refractor (Carl Zeiss Meditec, Inc., Miami, Florida, USA). Using the same autorefractor, post-cycloplegic measurements were recorded 20 to 30 minutes after instillation of one drop of tropicamide 1% in the eyes of participants over age 15 years and cyclopentolate 1% for participants younger than 15 years. Pre- and post-cycloplegic spherical equivalents were calculated using the standard formula of the algebraic sum of the dioptric powers of the sphere and half of the cylinder (sphere + 0.5 cylinder) (Toh et al., 2005a).

Intraocular Pressure

Prior to each examination session, the TONO-PEN XL (Reichert, Inc. New York, USA) was calibrated according to the manufacturer's recommended protocol. New custom latex covers were used on the instrument when obtaining IOP measurements for each participant. Topical anaesthesia with benoxinate was administered to each participant and he/she was then asked to fixate on a distant target. Sufficient measurements were performed to obtain a 5% reliability indicator on the instrument and this average reading recorded. The measurement time and order of eye tested was also recorded. In some adult cases IOP was measured with Goldmann applanation tonometry after instillation of benoxinate and fluorescein, with the examiner masked to the randomly set reading prior to measurement of each eye.

Pachymetry

Prior to each examination session, the pachymeter Tomey SP 2000 (Tomey Corp., Nagoya, Japan) and Pachmate DGH 55 (DGH Technology, Inc; Exton, PA, USA) was calibrated according to the manufacturer's recommended protocol. The pachymeter probe was disinfected with an alcohol swab between participants. Topical anaesthesia was administered to each participant and he/she was then asked to fixate on a distant target. Measurements were obtained from the central cornea and the average reading and standard deviation for each eye recorded.

Axial Length and Anterior Chamber Depth

The IOL Master is a non-contact ophthalmic instrument that provides accurate and repeatable ocular biometric measurements. Prior to performing the test, participant information (name, DOB) was entered into the device and the participant then positioned appropriately in front of the instrument. In device 'Overview' mode, a yellow fixation light is displayed and the participant was instructed to fixate on this to facilitate pre-testing alignment. Once the examiner was satisfied that both ocular alignment and device-to-eye distance was optimized (inspection of displayed corneal light reflections), the instrument joystick button was pressed and the first set of measurements (axial length) obtained. A similar procedure was then followed to acquire measurements of anterior chamber depth.

Eye Lash Measurement

Eye lash length was measured for each participant. After eye closure, a ruler was used to measure the longest observable lashes and this value recorded in millimetres.

Stereoscopic Fundus Photography

The Nidek 3-Dx fundus camera used in this study is based on simultaneous stereo photography principles and can be fitted with either a conventional film or digital camera back. Prior to performing photography, each participant's name and DOB was entered into a logbook recording details of each photograph taken and the initials and DOB entered onto the camera. This provides a method of cross-referencing photographs to the participants; for example, JS010190, John Smith 01/01/1990. In cases where twins had the same first and last initials, the middle initial was also used and a zero was deleted from the day or month. Alternative methods were used if all initials were the same or if dates were ambiguous; however, the coding format then remained consistent for future ease of comparison. To ensure errors in photo labeling were minimized, once the participant information was entered into the camera, identity was confirmed verbally.

The camera lens cover was replaced between photo sessions to avoid dust accumulation on the surface. The lens was examined for marks or dust before taking each set of photos and cleaned as necessary. After adequate pupillary dilation, participants were appropriately positioned in front of the stereo camera such that their forehead was in contact with the top

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headrest bar and their lateral canthus was at the height of optimized positioning.

When using the conventional camera back, the preferred slide film for photography was ektachrome 100. By experimentation, we found that flash exposure settings 2, 3 and 4 provided optimal exposure for this medium (compared to exposure settings 1, 2 and 3 for digital photography). Exposure settings were also adjusted after considering other factors such as poor dilation or variable fundus pigmentation. Six photos of each eye (varying exposure settings) were routinely taken to ensure at least one was adequately exposed.

Optic disc photos were taken by instructing the participant to fixate on the 'red light off to the side' (internal fixation light) and if this failed to center the disc, an external fixation target was used. Macular photos in older participants were acquired by asking the observer to fixate halfway between an upper and lower cross-shaped fixation lights.

Participants were verbally encouraged during their photo session with remarks such as 'Now you know what it's like to … be followed by the paparazzi … win the Brownlow Medal, etc.' They were also informed about the nature of the after-image and that it was a normal phenomenon.

Following each photo session, participant information was double-checked for accuracy. At the completion of each day or weekend's testing, log book entries and the day booking sheet were attached and returned to the main office in conjunction with the films for processing.

Participant Debriefing and Eye Health Report Card

Most twins are healthy volunteers, freely giving their time to genetic research. Providing feedback on the status of their eye health is an important benefit that researchers can provide in return and even emphasize that a normal baseline examination and photographs may be useful for the twins in later life.

Following completion of the examination session, each participant was given a detailed debriefing of the results of the various tests conducted, a summary of his/her ocular health status and provided with an opportunity to ask any questions. Common issues that frequently arose for discussion included:

- The difference between 20/20 and 6/6 vision.
- Why spectacles should not be prescribed based on autorefraction results.
- The potential benefits of baseline optic disc photos.
- The importance of regular eye examinations.

Zygosity Testing

Most twins were aware of their zygosity, although few had been genetically confirmed prior to our study. With the exception of different sex twin pairs (whose mothers always comment on being asked if the twins are identical), we conducted a least 10 microsatellite marker DNA testing through the Australian Genome Research Facility (AGRF) (Zhu et al., 2004). Queensland twins had previously undergone 700 marker microsatellite genome-wide linkage analysis and thus were clearly identified as DZ or MZ (despite some uneducated reviewers asking how we could be sure).

In most cases we confirmed the zygosity understanding of the family. However, the most common 'surprises' in zygosity occurred in MZ twins, who were clinically discordant for size, which had often been present from birth (although eye examination often indicated the true zygosity). Other causes of misinterpretation included the doctor telling the mother at birth that the twins were MZ or DZ (usually based on placental analysis). Despite overwhelming evidence to the contrary, parents stuck to what they had originally been told. Many parents thought they had been told that their twins were 99% identical (rather than the correct interpretation that we are 99% certain that they are 100% identical). All twins seen were given information about the study http://www.twinseyestudy.com/ and the ATR http://www.twins.org.au/. If they were not on the ATR, they were invited to enrol.

DNA Analysis

A subset of the BATS twins had previous 700 microsatellite markers data available for linkage analysis (Zhu et al., 2004). These results were used in some of our preliminary linkage analyses (retinal vessels and AL) (Sun et al., in press; Zhu et al., 2008). The majority of QIMR/BATS analysis involved Illumina 610 at deCODE. The majority of the TEST analysis involved Illumina 610 was conducted at the Center for Inherited Disease Research (CIDR). This latter analysis was funded by the NEI and there was a stipulation that phenotype and genotype data to be submitted to the National Institute of Health National Centre for Biological Information's genotype and phenotype database called 'dbGaP' (http://www.ncbi.nlm.nih.gov/sites/entrez?db = gap). Similar data were provided for twins from the parallel UK set. The phenotype data deposited onto dbGAP include: study site; study number; family ID; age; zygosity; sex; meanSphE; SphR CylR; axisR; SphL; CylL; axisL; IOPR; IOPL; CCTR; CCTL; C/D R and C/D L.

Additional Photographic Analysis Data

The Nidek 3-DX stereo images were digitized and analyzed with a stereo Z-screen (StereoGraphics, Beverly Hills, CA, USA) using high-resolution, 16-bit color images, high-speed modulating stereoscopic panel and passive polarized eyewear. The cup and disc were delineated with custom software (developed by James Morgan; Cardiff, UK) and modified for magnification using refraction and K readings. Further analysis of optic disc stereo photos, retinal vasculature and conjunctival auto-fluorescence are in process. UV photos measured with Adobe Photoshop.