Molecular Genetic Studies of Genes Predisposing for Glaucoma

BY

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Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Uppsala, Friday, May 7, 2004 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Glaucoma is one of the leading causes of visual impairment in the world. In glaucoma, the patient’s peripheral vision is lost due to progressive and irreversible deterioration of the retinal ganglion cells and atrophy of the optic nerve. The effect on the visual field is gradual and painless, and the progression so slow, that the patient may not notice until a substantial part of the visual field is lost. If left untreated, glaucoma can lead to blindness.

In this thesis, genes associated to glaucoma have been analysed in Swedish patients with primary open angle and exfoliative glaucoma. The genes studied were MYOC, oculomedin, GSTMI and OPTN.

The coding sequence of MYOC was analysed and mutations were found in 1% of the primary open angle glaucoma patients. Additionally, a predisposing variant was found in 1% of the patients as well as in 0.5% of the controls. No disease-associated variation was found in the exfoliative glaucoma cases. Mutations were also found in two families affected by glaucoma. The coding sequence of oculomedin was analysed, but none of the variants found were classified as disease causing in either patient group. GSTMI was analysed for its presence in the patients. No association could be found for either hetero- or homozygous deletions. The coding sequence and haplotype distribution of OPTN was analysed. None of the variants found were classified as disease causing and none of the haplotypes were associated to the disease in either patient group.

There are just a few per cent of the Swedish primary open angle glaucoma patients with genetic variation associated to disease, in the genes analysed in this study. No association to exfoliative glaucoma was found. This indicates heterogeneity in the genetics of glaucoma when different subtypes and different populations are compared. Likely, there are genes still to be identified.

Keywords: glaucoma, MYOC, OCLM, GSTMI, OPTN, mutation analysis

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ISSN 0282-7476
ISBN 91-554-921-8
urn:nbn:se:uu:diva-4142 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-4142)
"If we knew what it was we were doing, it would not be called research, would it?"
– Albert Einstein
Main references

This thesis is based on the following original publications.


IV  Mattias Jansson, Tayeh Rezaie, Mansoor Sarfarazi & Claes Wadelius. “Analysis of rare variants and common haplotypes in the optineurin gene in Swedish glaucoma cases” Archives of Ophthalmology, In progress

These papers are reprinted in the appendices at the back of this thesis. Reprints were made with the permission of the publishers.
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Abbreviations

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<td>Amplification-Refractory Mutation System</td>
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<td>cDNA</td>
<td>complementary DeoxyriboNucleic Acid</td>
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<td>dHPLC</td>
<td>denaturing High-Performance Liquid Chromatography</td>
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<td>Glutathione S-Transferase Mu One</td>
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<td>IOP</td>
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<td>Juvenile-onset Open Angle Glaucoma</td>
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<td>kb</td>
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<td>Normal Tension Glaucoma</td>
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<td>Optineurin gene</td>
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<td>PCAG</td>
<td>Primary Closed Angle Glaucoma</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>POAG</td>
<td>Primary Open Angle Glaucoma</td>
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<td>RNA</td>
<td>RiboNucleic Acid</td>
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<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<td>TIGR</td>
<td>Trabecular meshwork-Inducible Glucocorticoid Response gene</td>
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<td>Trabecular meshwork-Inducible Stretch Response gene</td>
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<td>TNFα</td>
<td>Tumour Necrosis Factor Alpha</td>
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<tr>
<td>UTR</td>
<td>Un-Translated Region</td>
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Introduction

Impaired vision, whatever the cause, is a significant handicap in today’s society. The cause of a persons impaired vision or blindness may vary considerably, be it accidents or disease, but a significant proportion of the cases are due to disease connected to genetic defects. One such disease is glaucoma.

Glaucoma mostly affects elderly people and is one of the most common causes of blindness in the world. It is a complex disease with both genetic and environmental factors contributing. The genetic component is illustrated by the fact that if a close relative have the disease, you have a greater risk of getting affected yourself.

The central symptom of glaucoma is loss of peripheral vision, which can progress to complete blindness. The disease progression is insidious in that the loss of vision is negligible at first and progress so slowly that the patient often does not notice until a substantial part of the visual field is lost (Figure 1).

*Figure 1. Comparison of the visual field as seen by a person with unaffected vision (left), and a glaucoma patient with severe loss of the visual field (right)*
The peripheral vision is important in many aspects of our daily life. Imagine taking a walk and constantly having to move your eyes or head from side to side, so you will not get run over or bump into people, because you will not see them out of the corner of your eyes. Imagine not being able to go outside at dusk or dawn, or move around a dimly lit room, because it is the peripheral vision, which enables you to see in poor light.

Once a person has lost parts of the visual field, it is irretrievably lost. Early detection and diagnosis is vital to stop the progression of the disease. To be able to do that, it is essential to gain more knowledge about the molecular genetic aspects of the disease.

In this thesis, I have studied selected genes associated to glaucoma for genetic defects. The identification of such defects may enable the development of new ways to diagnose and treat glaucoma.

Basic Molecular Genetics

DNA

In 1953, Watson and Crick discovered the structure of deoxyribonucleic acid, DNA (Figure 2). They established that the DNA strands are organised in a complementary double helix with specific pairing of the nucleotide bases, which in one go solved the problem of how an organism is able to copy its DNA.

Fifty years after the discovery of the structure of DNA, the complete human genome was published. The detailed order of some 2.8 billion base pairs, of the 3.2 billion base pairs estimated for the actual genome, are now accessible through the internet, together with an increasing number of annotations for genes, markers, polymorphisms and other features.

The DNA resides in the nucleus of each cell in the body. It consists of nitrogenous bases attached to a backbone of alternating deoxyribose and phosphate groups. The bases used in DNA are adenine (A), cytosine (C), guanine (G) and thymine (T). The order of bases in one strand of DNA is complementary to the bases in the other strand, i.e. A binds to T and G binds to C. Consequently, one strand can always be sym-
thesised with the other strand as a template; a process which is called semi-conservative replication.

The human DNA is divided into 46 chromosomes, with some additional DNA in the mitochondria outside of the nucleus. The human genome is diploid, i.e. there are two homologous copies of each chromosome. Each cell contains these 46 chromosomes except for the gametes, which contains a haploid genome with 23 chromosomes. There are 22 pairs of autosomes and one pair of sex chromosomes. The sex chromosomes determine the sex of the individual, where females have XX while males have XY.

**Genes**

The DNA is the blueprint for building, maintaining and operating an organism. A segment of DNA that contains the genetic information needed to code for a protein or an active ribonucleic acid (RNA) molecule, is called a gene. The gene is composed of coding sequence stretches called exons, with interspersed non-coding sequences called introns. Located prior to the first exon is the promoter, the region controlling expression and activity of the gene. Additional regulatory sequences are located in and around the gene. The information in a gene is keyed into the order of the bases. Three consecutive bases, called a triplet, codes for a specific amino acid, or codes for a start or stop in the protein synthesis. An amino acid, of which there are 20, is the basic component of a protein.

Using the gene as a template, an RNA molecule is synthesised in a process called transcription. The difference between RNA and DNA is that in RNA, thymine is exchanged for uracil (U) and the backbone consists of alternating ribose and phosphate groups. The RNA is then modified into messenger RNA (mRNA), by splicing out the introns, and the connected exons are then transported out of the nucleus into the cytoplasm of the cell. The mRNA is then used as a template for protein synthesis in a process called translation. The protein can then be further modified into a finished product.

These proteins are then responsible for the regulation, synthesis and maintenance of other types of molecules and structures needed in the organism, including the copying and maintenance of the DNA and the machinery necessary to transcribe and translate the genetic information.

Thus far, 25-30,000 genes coding for proteins have been predicted in the human genome, which, using alternative promoters and splicing, would result in an estimated 300,000 different proteins, supplied in the right quantities, at the right times, and in the right places. In addition, there are perhaps 10-15,000 genes not coding for proteins, i.e. the RNA is the final product. Some of them are well known and are called non-coding RNA (ncRNA), e.g. rRNA, tRNA or snRNA.

Most of the genetic material in the genome does not code for proteins or ncRNAs, but consists of repetitive sequences and sequences often called
“junk DNA”. The functions of these sequences are unknown, but they could have important structural and/or regulatory properties.

A major challenge for the last few years and the years to come is to decipher the biological information encoded in the DNA as well as how the genes and proteins function, how they are regulated, and how they work together to carry out complex biological processes.

Genetic Variation

No two individuals are exactly the same at the genetic level, not even monozygotic twins. These differences at the genetic level are commonly called mutations or polymorphisms, and are studied in molecular genetic analysis of disease. Most of this variation (in genotype) is not associated to disease or to any physical (phenotypic) properties in any way. Most variants that are, are associated to a phenotypic trait such as height, looks or the consistency of earwax. Some, however, confer susceptibility to disease.

An individual’s genome may differ from that of another individual in numerous ways. These differences can range from chromosomal rearrangements, through insertions, deletions and translocations, down to single base substitutions. The single most common type of polymorphism in the genome is the point mutation, or single nucleotide polymorphism (SNP). An SNP is a substitution of one single base for another base. The definition of an SNP stipulates that the least frequent allele have to be present in at least 1% of a population; otherwise, it is defined as a rare variant.

Depending on context, these substitutions can interfere with normal functions in the genome. For example, if the variant is located in the coding sequence of a gene, it may result in an exchange of the proper amino acid, altering the synthesis or function of the protein. If the variant is located in the promoter region, it may change the binding site of a regulatory factor, thereby changing the transcription level of the gene. Most variation, though, is located in between genes, and has no functional properties.

Estimates show that each individual compared with another individual, have one SNP on average every 1000 bases, resulting in several million SNPs for the entire genome. Additionally, the DNA in our own germ cells undergoes an estimated 100 new mutational events per generation, through mistakes during either DNA copying, cell division or because of damage from the environment. These variants are then passed on to our offspring.

A mutation is a process through which the DNA sequence is changed. The term mutation or mutant, though, is often used to refer to (disease-causing) variant alleles as opposed to the (normal) wild type. When the mutation increases in frequency in the population, it is instead referred to as a polymorphism. Usually, a polymorphism is regarded as a normal variant, while a mutation is regarded as connected to disease; a distinction not always correct.
The difference between individuals is not solely due to genetic variation. The effects of the environment and the expression levels of crucial genes at precise moments during the development are also important factors.

Monogenic disease

In 1866, Gregor Mendel published his theories on the inheritance in plants (translated *Experiments in Plant Hybridization*, 1926). Mendel postulated the existence of hereditary units, which give rise to certain characteristics, or phenotypic traits, following strictly segregating inheritance patterns from one generation to the next. Mendel’s “principle of segregation” states that everyone inherits two hereditary units for each trait, one from each parent. Slightly different versions of the hereditary units confer different phenotypes of a trait to the organism, e.g. smooth or wrinkled skin on peas. During meiosis, these units separate equally into the gametes, which carry only one unit. Mendel’s “principle of independent assortment” states that hereditary units for different traits are inherited independently of each other.

Mendel also discovered that some versions of a hereditary unit, or allele, always “dominate” over other “recessive” alleles. A simplified but classic example of this is that the allele for brown eye colour dominates over the allele for blue eye colour. This means that if an individual has one “brown eyes” allele, that individual will get brown eyes, regardless of what the other allele is. On the other hand, an individual needs two “blue eyes” alleles to get blue eyes. In this case, the “brown eyes” allele is dominant and the “blue eyes” allele is recessive. In monogenic disorders, the situation is the same. Either one allele is enough to get the disease, i.e. a dominant disease, or two alleles are needed, i.e. a recessive disease.

Although a good deal of these laws has been modified and updated, the fundamental ideas are still correct and are the basis of modern genetics. The hereditary units are now commonly referred to as genes, and the slightly different versions of a gene are called alleles. An allele is defined as an exclusive form of a gene, occupying the same chromosomal position and governing the same biochemical process.

Most genetic disorders discovered to date are monogenic and follow a simple Mendelian inheritance pattern. In a monogenic disease the phenotype is caused by an abnormality in one single gene, which may contain a point mutation or an insertion/deletion that changes the coding sequence or promoter, and hence the amino acid sequence or expression profile of the protein, thereby triggering the disease.

Although environmental factors, age of onset and/or allelic heterogeneity (several different alleles of the same gene, giving rise to the same disease phenotype) can complicate the picture, monogenic disorders usually have a high correlation between genotype and phenotype i.e. there is a high penetrance. The penetrance is a measure of the probability that a person carrying
a specific genotype (variant allele) is expressing the disease (displays the phenotype).

A monogenic disease with a severe phenotype would be expected to diminish in a population due to negative selection. Unaffected carriers and selection in favour of heterozygotes may contribute to keep recessive disorders in a population, but dominant disorders are under a stronger evolutionary pressure. A high recurrent mutation rate in a gene may be responsible for maintaining a dominant disease in a population. Alternatively, if the dominant disease has a late age of onset, the carrier will have had time to transmit the susceptibility allele to his or her offspring before the onset of disease. Another possibility is variable expressivity, where the same genotype produces different phenotypes of variable degree of severity.

An example of a disease with a recessive mode of inheritance is Cystic Fibrosis. The disease is caused by mutations in the $CFTR$ gene. Approximately 70% of the cases are caused by the delta-F508 deletion, but there are over 550 additional rare mutations reported, making this an example of allelic heterogeneity. An example of a disease with a dominant mode of inheritance is Huntington’s disease. This disease is caused by an expansion of a CAG triplet in the $huntingtin$ gene. Huntington’s disease has a severe phenotype, but the late age of onset maintains the variant allele in the population.

Complex disease

While there are a plethora of monogenic disorders, they usually only affect relatively few people in a population, albeit often with severe phenotypes. Complex diseases, on the other hand, are much more common and affect millions of people worldwide. For example, complex disease includes disorders such as rheumatoid arthritis, diabetes and glaucoma. Complex diseases usually do not segregate in families in a Mendelian fashion, although they tend to aggregate in families. As many complex disorders, glaucoma is exhibiting both Mendelian and complex inheritance.

A monogenic disease is, per definition, dependent on abnormal alleles in one single gene, whereas a complex disease has multi-factorial inheritance, i.e. the aetiology of complex disorders is dependent on the interactions between any number of different genes and/or environmental factors. Usually, there is no single gene or environmental factor that is, in itself, either sufficient or necessary for the disease to manifest. The manner in which these genes and environmental factors interact can be additive, interactive or epistatic.

Complicating the picture is the fact that the allelic variants predisposing for a disease are often common variants found in a large part of the healthy population. These people may live out their full life span without being affected by the disease they in fact carry susceptibility for. The reduced or
incomplete penetrance in these people is influenced by age of onset, sex, environmental factors and genetic background.

An accurate diagnosis is vital for the identification of susceptibility genes for a complex disease. One complicating factor in the identification of genes and alleles associated to disease is locus heterogeneity, in which the disease is dependent on genetic factor/s in different loci, with the same indistinguishable phenotype. Another complicating factor is phenocopies, where the disease is caused solely by environmental factors.

Identification of genes associated to complex disorders by conventional means, such as mapping of linkage in families, has often been futile since the signal is scattered over several loci each contributing to the disease in a small way. An alternative way to finding genes for complex disease is case-control studies of candidate genes. A candidate gene is selected based on a priori hypotheses of their role in the disease.

An interesting ongoing debate discusses theories concerning common, complex disease. The first theory is that genetic risk is due to interactions between a few genes with a few common variants, called the “common disease-common variant” hypothesis. The rivalling theory is that rare alleles in numerous genes each single-handedly cause the disease. Neither one theory is probably correct, but there is rather an amalgam of the two.

Glaucoma

Glaucoma is one of the most common causes of visual impairment and blindness in the world. Estimates of the number of glaucoma cases worldwide range from 67 million (The Glaucoma Foundation, 2001) to 105 million "suspect" cases (World Health Organization, 1997c). About eight million of these cases are clinically blind. The incidence of glaucoma increases with age, with a prevalence of about four per cent at the age of 75. Prior to the age of 50, the disease is uncommon.

Glaucoma is a collection of diseases with certain symptoms in common. The fundamental characteristic is the progressive and irreversible degeneration of the retinal ganglion cells with a corresponding deterioration of the optic nerve and cupping of the optic nerve head. The cupping of the optic nerve head is today the most important diagnostic criterion in the diagnosis of new glaucoma cases. The symptoms noticeable to a patient is not only a gradual reduction in the visual field, they can also experience headache, nausea, rapid reduction of the visual field, halos around light sources and pain.

In many cases, glaucoma is accompanied by an elevation of the intraocular pressure (IOP), but whether this should be considered a diagnostic criterion or a risk factor is under debate. Reduction of the IOP has been found to
slow the progression of glaucomatous damage, and this is at present the only treatment available\textsuperscript{11}.

Glaucoma is subdivided depending on the presence of primary and secondary characteristics. Primary characteristics include the status of the iridocorneal angle and the age of onset. Secondary characteristics include characteristics such as IOP, pseudoexfoliations and developmental abnormalities.

- Primary Open Angle Glaucoma (POAG) – Primary type of glaucoma with late age of onset, open iridocorneal angle and elevated IOP.
- Normal Tension Glaucoma (NTG) – Primary type of glaucoma with open iridocorneal angle and normal IOP. This type of glaucoma is sometimes sorted under POAG.
- Juvenile onset Open Angle Glaucoma (JOAG) - Primary type of glaucoma with age of onset between three and forty years of age, open iridocorneal angle and elevated IOP.
- Primary Closed Angle Glaucoma (PCAG) - Primary type of glaucoma with closed corneal angle and elevated IOP. Acute form of glaucoma.
- Congenital Glaucoma - Primary type of glaucoma with onset prior to three years of age, open iridocorneal angle and elevated IOP.
- Secondary Glaucoma – A conglomerate of forms with a secondary cause such as pseudoexfoliations, developmental abnormalities or trauma.
- Exfoliative glaucoma – Secondary type of glaucoma with late age of onset, open iridocorneal angle, elevated IOP, and pseudoexfoliations.

The genetic contribution to the risk of glaucoma has been estimated by studying first-degree relatives (either a parent, a sibling or a child) of glaucoma cases. A first degree relative of a POAG patient has a 7 to 10 fold increase in the risk of developing glaucoma themselves compared to a person in the general population\textsuperscript{12}.

Structures in the eye with relevance to glaucoma
The principal symptom in glaucoma is the degeneration of the retinal ganglion cells in the retina, with the corresponding atrophy of the optic nerve (Figure 3). The retinal ganglion cells convey the signals from the light responsive photoreceptors in the eye, to the visual centres of the brain. The axons of the ganglion cells leave the eye through the optic nerve via the optic nerve head. The primary indication and a diagnostic criterion of glaucoma is the cupping, or excavation, of the optic nerve head, which occurs when the retinal ganglion cells are degraded.
The bulk of the matter in the eye consists of the gelatinous vitreous humor. In addition to this, the ciliary body produces aqueous humor, a fluid that transports oxygen and nutrients to the cells in tissues lacking blood vessels, such as the lens and the cornea. To facilitate a flow of liquid and to prevent a continuous increase in the amount of fluid in the eye, the aqueous humor is drained from the eye.

The primary mean of aqueous outflow from the eye is through the conventional (trabecular) outflow mechanism. The trabecular meshwork (TM) is a structure located in the angle between the cornea and the iris. The aqueous humor filters through the meshwork into Schlemm’s canal, which empties into the venous system through sinus venosus sclerae. The TM is proposed to be involved in the regulation of the outflow, but the mechanism by which this occurs is unknown. The unconventional (uveoscleral) outflow mechanism is a secondary means of aqueous outflow from the eye. The uveoscleral outflow essentially takes place through the muscle bundles of the ciliary body, and consists of about 10% of the total capacity at normal pressures.

Figure 3. Structures in the eye with relevance to glaucoma
Glaucoma associated loci

Full genome scans in glaucoma families have so far revealed eight loci, or chromosomal regions, with significant linkage to glaucoma. These are GLC1A in 1q24.3-q25.2, GLC1B in 2cen-q13, GLC1C in 3q21-q24, GLC1D in 8q23, GLC1E in 10p15-p14 and GLC1F in 7q35-q36 for adult and juvenile onset glaucoma\textsuperscript{13-18}, and GLC3A in 2p22-p21 and GLC3B in 1p36.2-p36.1 for congenital glaucoma\textsuperscript{19,20}.

The first gene identified in these loci, the myocilin gene (MYOC), located in GLC1A, is associated to glaucoma in families with JOAG and a subset of patients with POAG\textsuperscript{21}. The second gene, CYP1B1, located in GLC3A, is associated to primary congenital glaucoma\textsuperscript{22}. The latest gene, optineurin (OPTN), in locus GLC1E, is associated to families with primarily NTG\textsuperscript{23}. 
Aims of the studies included in this thesis

The main aim of these studies was to evaluate selected candidate genes reported as associated to glaucoma, in our cohorts of Swedish POAG and exfoliative glaucoma patients. Given the complexity of the genetics and the different phenotypic subgroups of glaucoma, it is not possible to predict whether a gene associated to one subgroup, is also associated to other subgroups.

The *MYOC* gene has been established as associated to glaucoma in JOAG and POAG cases for some time. Our aim in this study was to evaluate the previously reported associations of this gene to POAG and find a possible association to exfoliative glaucoma, a subgroup previously not analysed for this gene.

The *oculomedin* gene was reported as a candidate gene for glaucoma due to its induction in TM cells submitted to stress mimicking the increase in IOP common in glaucoma. The aim of this study was to find alterations in the coding sequence in our patient cohorts, to determine if this gene was in fact associated to glaucoma.

The *GSTM1* gene has the unusual characteristic of being deleted in a percentage of humans. The gene was reported to be more frequently present in glaucoma patients. The aim of our study in this case was to evaluate this report and to analyse the level of deletion in our cohorts. In addition, we wanted to evaluate the possible difference between hemi- and homozygosity.

The *OPTN* gene has been implicated in families with mainly NTG, and a subset of sporadic cases of NTG, in addition to possible high IOP cases. To further our understanding about the association of *OPTN* to different subgroups of glaucoma, our aim was to screen the *OPTN* gene in our patient cohorts. In addition, we wanted to construct haplotypes spanning the entire gene by the use of SNPs to increase our chances of finding associations.
Subjects and methods

Subjects

Recruitment
The patients included in these studies were recruited from the out-patient clinics at the Department of Ophthalmology, University Hospital, Uppsala and the Department of Ophthalmology, Tierps Hospital, Tierp. The patients were each informed as to the intentions of these studies and gave their consent to be included. Samples of peripheral blood were collected from 200 patients diagnosed with POAG, as well as 200 patients diagnosed with exfoliative glaucoma. All patients were unrelated and randomly chosen from the clientele of the out-patient clinics.

These studies have been approved of by the Uppsala University Research Ethics Committee (#96481), and were performed according to the Declaration of Helsinki.

Diagnosis
The diagnostic criteria used for patient recruitment were increase in the IOP, and glaucomatous damage to the optic nerve head and/or glaucomatous damage of the visual field. Regarding the exfoliative glaucoma patients, the additional criterion of presence of pseudoexfoliative material on e.g. the iris or lens was required for diagnosis.

Diagnoses were obtained from the patients’ permanent medical records. The grading of the optic nerve head damage was performed by the patients’ treating and examining physician. Correspondingly, different perimetric methods for visual field testing were used, and the grading of the visual fields was based on the examining physicians’ evaluation. At the two clinics involved, dilatation of the pupils and gonioscopy are standard procedures for the diagnosis of glaucoma.

Concerning the gender distribution, it was found to be equal in the POAG cases, whereas in the exfoliative glaucoma cases, 114 patients were female and 86 patients were male. The mean age at diagnosis was 65.8 and 69.2 years, and the mean age at the collection of samples was 76.5 and 79.6 years in the POAG and exfoliative glaucoma cohorts, respectively. The mean IOP at diagnosis was 32.9 mmHg in the primary open angle glaucoma cases and 38.7 mmHg in the exfoliative glaucoma cases. At the latest examination of
the patients, there were significant damage to the visual field and optic disc in both groups.

**Controls**

To aid in the detection of new disease-associated variants, 200 unrelated controls were recruited. These controls were matched for age, sex, and geographic and ethnic origin. Glaucoma was excluded in all controls by measurements of the IOP and ophthalmoscopy of the optic disc. The controls gave their informed consent to be included in the studies.

To evaluate and to test the mutation scanning techniques before the analyses, one affected person from each of two families segregating JOAG were utilised as positive controls. The first family is Danish with 21 affected members available for analysis. They had an early age of onset with a mean at 18 years, range 7 to 33 years, with only two being 25 years of age or above. Their IOP was high and varied between 30 and 59 mmHg, and at diagnosis, several had severe damage to the optic disc and visual field. Linkage studies showed significant linkage to the 1q21-31 genetic region, with a maximum LOD score of 6.67 for marker D1S210. The second family was smaller, with a clear autosomal dominant pattern of inheritance. Both families carried mutations, which segregated with the disease.

**Methods**

**Polymerase Chain Reaction**

During a night time car ride in 1983, Kary Mullis invented a method, which would revolutionise molecular biology; polymerase chain reaction (PCR). The technique allows a scientist to quickly make millions of copies of a DNA fragment.

PCR utilises the thermostable DNA polymerase (DNA polymerases copies DNA in most organisms) of the bacterium *Thermus aquaticus* to withstand the temperature changes inherent in the method. A single stranded DNA molecule has the ability to find and bind specifically to a complementary sequence. Careful design of oligonucleotide primers flanking the desired sequence is utilized to specifically amplify one unique fragment from a whole genome. A cyclic amplification process gives an exponential increase in the number of copies.

PCR is now invaluable to scientists enabling them to multiply unique regions of DNA, to facilitate subsequent detection and analysis.

**Amplification-Refractory Mutation System**

Building on the PCR methods’ specificity in amplifying discrete fragments in a genome, the amplification-refractory mutation system (ARMS) is used
to differentiate between alleles of a specific polymorphism, usually an SNP. This differentiation is achieved by synthesising two oligonucleotide primers with the 3’-nucleotide in the variable position, specific for either the normal or the variant allele. When the analysis is run, separate reactions are made for each of the two allele-specific primers. If amplification occurs, the sample contains the allele in question\(^2\). Usually, to increase the specificity of the analysis the third nucleotide from the 3’ end of the oligonucleotide is changed to decrease the hybridisation strength of the unmatched primer.

This and similar methods are often referred to as allele-specific PCR. The method is useful for scanning a large number of patients for a specific variant to e.g. find out the frequency of the allele in a population.

**Sanger sequencing**

Sanger sequencing is today the most common method used for sequencing DNA fragments of up to 800 bases. The method uses DNA polymerase to synthesise products from the template strand. With the use of fluorescently labelled nucleotide terminators mixed with the normal nucleotides, the copying process of a fragment is stopped when incorporation of a terminator nucleotide occurs. The result is product fragments of all sizes, with a fluorescently labelled nucleotide at the end. The colour of the fluorophore corresponds to the nucleotide in the original sequence. The fragments can then be separated according to size on a slab-gel or in capillaries, and analysed using a computer.

This type of sequencing is used when the specific order of all bases in a sample fragment is needed, e.g. to identify a variant detected in another unspecific method.

**Single base-pair extension**

To sequence only the position of interest, e.g. a variable position, single base-pair extension can be used. One such method is SNaPshot.

In SNaPshot, internal primers are hybridised to positions right next to polymorphic sites in a PCR fragment. A sequencing step attaches one fluorescently labelled nucleotide terminator to the variant position and the resulting labelled primer is analysed as in Sanger sequencing. By attaching oligonucleotide tails of different length to the internal primers, multiplexing of up to ten variant sites in each reaction is possible.

**Pyrosequencing**

A sequencing method based on radically different chemistry is Pyrosequencing.

When a nucleotide is incorporated into the product fragment in a sequencing reaction, a pyrophosphate is released. The energy in the pyrophosphate can be utilised by an enzyme to produce a flash of light. The intensity of the peak corresponds to the number of bases incorporated. By adding nucleo-
tides in a predetermined order and detecting the light peaks, the order of bases in the template fragment can be determined.\textsuperscript{27} Pyrosequencing is used primarily for typing of known variants or sequencing of shorter fragments.

**Denaturing High-Performance Liquid Chromatography**
Since the 80’s, high-performance liquid chromatography (HPLC) has been used for oligonucleotide purification and analysis, and in 1994, the method was refined to allow separation of heteroduplexes from homoduplexes near the melt temperature. Denaturing HPLC (dHPLC) was found to be efficient in the discovery and screening of SNPs, insertions, deletions and repeats.\textsuperscript{28}

In dHPLC, an ion-pairing agent is allowed to bind to the DNA fragment. This agent compresses the melting range of the fragment, enabling the separation of fragments according to length at non-denaturing temperatures. Since the ion-pairing agent binds more efficiently to double-stranded DNA than to single stranded DNA, a partially denatured fragment binds less of the agent. This is utilised by the creation of heteroduplexes, in which there is a mismatch in one position of the fragment, by mixing a mutated sample with a wild type sample, then denature and re-anneal the mix. The mismatch will create an area of reduced thermostability in the fragment. At a specific temperature, the heteroduplexes will start to denature while the homoduplexes will not. The separation column binds DNA fragments according to how much ion-pairing agent is bound to the fragment. The specific temperature is achieved by placing the separation column in an oven. The denatured fragment retains less ion-pairing agent and is eluted earlier in a gradient of organic solvent.

In dHPLC, design of PCR products to obtain an even melting range throughout the entire fragment is vital to achieve a high frequency of detection.

DHPLC is useful for scanning large numbers of samples for unknown sequence variants. A drawback of dHPLC is that the method does not identify the specific variant, only that there is a polymorphism in the fragment.

**Enzymatic mutation detection**
Enzymatic mutation detection (EMD) utilises the property of an enzyme to detect and digest a conformational irregularity in a fragment of DNA.

Passport is based on the EMD technology.\textsuperscript{29} In Passport, a fluorescently labelled PCR fragment is denatured and re-annealed to create fragments with possible mismatches. Homozygous mutants can be analysed by addition of a wild type sample prior to denaturation. The mismatch will create a conformational irregularity, a “bubble”, in the fragment. The enzyme Endonuclease VII is then used to digest the fragment. If the subsequent size separation analysis reveals additional fluorescent peaks, indicating fragments of alternative lengths, a heterozygous mutation is present in the sample.
Results and discussion

Paper I: Allelic Variants in the MYOC/TIGR Gene in Patients with Primary Open Angle, Exfoliative Glaucoma and Unaffected Controls

Background
In 1993, Sheffield et al. identified the first locus associated to glaucoma, GLC1A, at chromosomal location 1q21–q31. The locus was further fine mapped to 1q24.3–q25.130.

In 1997, Kubota et al. and Polansky et al. independently cloned the gene, and called it myocilin gene and trabecular meshwork inducible glucocorticoid response (TIGR) gene, respectively31,32. The Human Genome Organization Genome Database Nomenclature Committee later assigned the terms myocilin for the protein and the gene symbol MYOC for the gene. Stone et al. eventually identified causative variants in the MYOC gene in JOAG patients verifying the association to glaucoma21.

The three exons of MYOC translate into a protein consisting of 504 amino acids with a molecular weight of 57 kDa. The protein exists in a glycosylated as well as a non-glycosylated form31,33. Myocilin is expressed in most parts of the eye, such as the TM, cornea, retina and the optic nerve, as well as several other tissues. Depending on the tissue, it is located intra- or extracellular34.

The function of the myocilin protein is unknown, but patients with mutations in the gene usually have a higher IOP than those with other forms of POAG21,35,36. This phenotype indicates that mutated myocilin interferes in some way in the aqueous humor outflow through the TM. Whether wild type myocilin plays a role in the aqueous outflow during normal conditions is less clear. Myocilin seems to be multifunctional, as it has been shown to be associated to mitochondria in TM cells37, to fibronectin and fibrillin-1 in the trabecular extra cellular matrix38,39, and is present in the aqueous humor as homomultimer complexes40-42.

Mutated alleles of MYOC are primarily found in families with JOAG but also to a subset (1–4%) of POAG cases21,36,43-60. Numerous additional disease-associated mutations have been identified in the gene, mostly in families segregating glaucoma30,61-85. Of the disease-associated changes identified
in MYOC, most are clustered within the third exon, while few are located in the first and second exon\(^{43,81}\).

**Results and discussion**

As the oldest known glaucoma associated gene, MYOC has been studied in most types of populations. Prior to this study, though, the gene had not been studied in a Swedish population or in a large exfoliative glaucoma population.

In this study, we screened our 200 POAG and 200 exfoliative glaucoma patients, and 200 controls using EMD, ARMS and dHPLC, for sequence alterations in the *MYOC* gene. We identified a total of eight sequence alterations, of which two were considered disease-causing mutations, and one a disease-associated variant. All disease-causing mutations were found in the POAG cases, indicating a prevalence of 1% in this patient group within the Swedish population. This frequency is below that which has been reported in other populations, but not significantly so. No strictly defined (see below) disease-causing mutations were found among the exfoliative glaucoma cases, indicating a fundamentally different genetic origin of glaucoma in this patient group.

The criteria we used to define the disease-causing status of an allele were based on genetic evidence. In essence this meant that the variant allele had to be absent from our carefully selected controls and that it altered the size, charge and/or polarity of the amino acid. In general, such a protocol has a high probability of discriminating between disease causing and neutral alleles. To provide proof that a variant allele is causing disease, a functional test is required. However, a verified test regarding myocilin is not yet available.

Two variant alleles were found which adhered to the criteria used to define causality. The first, T285M, causes a change in polarity from the neutral and polar threonine to the hydrophobic methionine. The second, P481R, changes the neutral and hydrophobic proline to the basic residue arginine, a change that is predicted to have a major effect on the protein, in that the bend in tertiary structure induced by proline may be lost. P481T and P481L have been identified in US glaucoma patients\(^{43}\). These changes are considered disease causing, which supports our status of the P481R change.

The Q368X variant is a bit more problematic. We identified the allele in two POAG cases and in one control, which makes it questionable if this allele should be classified as disease-causing, although it was first reported as such\(^{43,75}\). The variant has a reduced penetrance and a milder phenotype and could more appropriately be regarded as predisposing change or a phenotypic modifier rather than a variant that is causing glaucoma\(^{86,87}\). This variant is the most common single variant in most populations studied\(^{43,45,62,75,87,88}\). Interestingly, it has not been found in Asian populations\(^{43,44,58,59,65,66}\). Its presence in African-Americans is unusual and probably produced by admix-
ture. This variant allele is usually reported as disease causing, and is likely the reason why our estimate of the frequency of disease-causing mutations is lower than in many other populations.

The remaining variants found in this study were conservative (R76K, D208E, K398R) or synonymous (T285T, V439V). An additional variant we identified was T256M. It was found in a control subject and would have been classified as disease causing, had it been found in a glaucoma case, as it changes neutral and polar threonine to the hydrophobic methionine.

To obtain mutations for use as positive controls to test the methods, we sequenced members from two Danish glaucoma families. We identified the novel variants T438I and P370L, both of which segregated with the disease in their respective family, and met our criteria for disease-causing mutations.

Most disease-causing point mutations do not compromise the synthesis of the protein, but rather the protein folding and stability of the folded protein. If the folding is impaired, the resulting mis-folded protein is usually degraded by cellular control mechanisms, or failing that, retained in the ER. Zhou et al. proposed a functional test in which the solubility of mutant myocilin in Triton X-100 is reduced compared to wild type myocilin. The reduction of solubility could be correlated to the seriousness of the glaucoma phenotype resulting from the specific disease-associated mutation. The reduced solubility could explain possible retention of mutant myocilin in the ER. Studies by Jacobson et al. have revealed a reduction in the secretion of mutant myocilin, as well as suppression of normal myocilin secretion in co-transfected cells. The following congestion of the secretory pathways may be deleterious to the cells in the TM.

Autosomal dominant disorders may have a more severe phenotype in homozygotes than in heterozygotes, or at least have an average disease phenotype. Interesting findings suggest that this might not be the case in glaucoma. Morissette et al. reported asymptomatic members of a French-Canadian pedigree, which were homozygous for the mutated allele.
Paper II: Evaluation of the Oculomedin Gene in the Etiology of Primary Open Angle and Exfoliative Glaucoma

**Background**
An increase in the IOP is a common characteristic in many subgroups of glaucoma. Consequently, genes involved in, or induced by the increase in pressure are obvious candidate genes for the disease. By subjecting cells from the eye to stress in the form of mechanical stretching, mimicking the mechanism of the disease, it is possible to compare the expression of proteins before and after the stress response. The differences in the expression pattern allow the finding of possible candidate genes involved in the response to that particular stress.

Sato *et al.* subjected TM cells to cyclic mechanical stretching. One gene found to be induced by this was the *oculomedin*, or the trabecular meshwork-inducible stretch response (*TISR*) gene. The *oculomedin* gene is located in 1q25.3 and consists of only one exon. Other features are an Alu-repeat in the 5'-untranslated region (UTR) and a sequence homology to neuromedin K. The gene product is a small protein of 44 amino acids, of unknown function. The gene is expressed in the TM and in the retina, but expression was not detected in the brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung or peripheral blood leukocytes.

Leung *et al.* investigated the coding sequence and promoter region of *oculomedin* in a cohort of Chinese POAG patients, but found no suspected disease associated alterations. Fujiwara *et al.* identified amino acid altering nucleotide substitutions in two of 75 POAG patients. They also discovered that *oculomedin* is expressed only in stretched TM cells and not in control TM cells. In addition, they located the protein to the TM, Schlemm’s canal endothelium, photoreceptor cells, and corneal and conjunctival epithelium.

**Results and discussion**
In this study, we used dHPLC to screen our 200 POAG and 200 exfoliative glaucoma cases, and 200 controls for allelic variants in the *oculomedin* gene. This gene is ideal for analysis on the dHPLC; there is only one small exon, and the melting profile of the exon is even.

We found no association between alleles in the *oculomedin* gene and glaucoma in neither of the patient groups, or the controls. This is in concordance with the study by Leung *et al.*, in which no mutations were found in the *oculomedin* gene in Chinese POAG patients.
Changes identified in our study were 104C>G, which was found once in each patient group as well as twice in the controls, 154A>G in the 3′-UTR of the oculomedin gene in an exfoliative glaucoma patient, and -33T>C in the 5′-UTR of the gene in two controls.

The only change found that affected the coding sequence, 104C>G, does not seem likely to be causative of glaucoma, since the change was found in both patients and controls. Never the less, it changes the uncharged polar serine to the likewise uncharged polar cysteine. This change gives no difference in charge, polarity or size, but it does change the hydroxyl group of serine to the sulfhydryl group in cysteine, and creates the possibility of a disulphide bond. This could change the folding, and thereby the function of the protein.

On a side note, we noticed that the sequence of the gene in the Swedish population differs from that of the cDNA posted in the NCBI database. Our sequence contained the synonymous change 84G>A (K28K) and the deletion 135+36delC in the 3′-UTR in all patients and controls screened. Leung et al. reported the same sequence variants\textsuperscript{94}. However, there were no differences compared to the \textit{Homo sapiens} chromosome 1 working draft sequence, implying sequencing mistakes in the cDNA sequences posted prior to the working draft in the NCBI database.

Since the expression level of this gene is what lead Sato \textit{et al.} to suggest it as a possible glaucoma candidate gene, more extensive studies of the promoter region could be performed. However, in a similar way as with the also inducible \textit{MYOC} gene, causative mutations could have been present in the coding sequence.

**Paper III: Analysis of the Glutathione S-transferase M1 gene using Pyrosequencing and multiplex PCR–no evidence of association to glaucoma**

**Background**

For an organism to be able to cope with harmful compounds, there are specialised gene families, like the glutathione s-transferase (GST) gene cluster, that is involved in the elimination of hazardous substances. These substances also include compounds that we introduce into our own bodies for their beneficial properties, such as medicine. The level at which a drug is metabolised is important regarding our tolerance to medical treatment and the dosage thereof.

A subclass in the GST cluster, the mu class 1 gene (\textit{GSTM1}) has been associated to glaucoma in an Estonian study\textsuperscript{96}. The gene has three major alleles, one of which, the \textit{GSTM1}\textsuperscript{0} is a total deletion of the gene. The other
two, \( \text{GSTM1}^*A \) and \( \text{GSTM1}^*B \), differs only in the amino acid K172N and seems to have equivalent enzymatic function\(^97,98\).

Juronen et al. found that the frequency of \( \text{GSTM1}^*0 \) individuals were decreased in glaucoma patients as compared to the general Estonian population. Their conclusion was that carriers of a functioning \( \text{GSTM1} \) gene copy had a higher risk of developing glaucoma\(^96\). Western blot analysis performed by Yang et al. revealed that serum antibodies against GST antigen were detected in 52% of the glaucoma patients and 20% of age-matched control subjects. This gives indication of an autoimmune involvement in glaucoma\(^99\). Izzotti et al. presented contradicting evidence. They found that oxidative DNA damage is significantly increased in the TM of glaucoma patients, and that the \( \text{GSTM1}^*0 \) genotype is significantly more common in glaucoma patients than in controls. They suggest that \( \text{GSTM1}^*0 \) are predisposing to a more severe oxidative DNA damage in glaucoma patients\(^100\).

**Results and discussion**

The significance of one or two copies of the \( \text{GSTM1} \) gene has not been investigated before, mostly due to the difficulty in assessing the number of copies. By utilising Pyrosequencing we were able to distinguish between people who were hemi- and homozygous for the presence of the \( \text{GSTM1} \) gene. Using multiplex PCR and Pyrosequencing, we scanned our 200 POAG and 200 exfoliative glaucoma cases, and 200 controls for the presence of either 0, 1 or 2 copies of the \( \text{GSTM1} \) gene, taking advantage of differences in the sequence between \( \text{GSTM1} \) and \( \text{GSTM4} \). The \( \text{GSTM4} \) gene in this case used as a positive control for dose. In almost all cases it was not only possible to unequivocally determine the presence or absence of the \( \text{GSTM1} \) gene, but also to establish if the person carried one or two copies of the functional \( \text{GSTM1} \) gene.

In our Swedish cohorts, there was no significant difference between the number of cases and the number of controls that were positive for the gene. The frequency of \( \text{GSTM1} \) positive cases was 44.0% in POAG and exfoliative glaucoma, and 44.5% in the controls as determined by multiplex amplification genotyping. The frequency of \( \text{GSTM1} \) positive individuals in our study agrees well with data from a meta analysis of several European populations where 46% of normal European populations were found to be positive (M. Wadelius, personal communication). Five per cent of the POAG patients, 7.4% of the exfoliative glaucoma patients and 4.6% of the controls were homozygous, i.e. carried two copies of the \( \text{GSTM1} \) gene. There was no significant difference between the patient groups and controls regarding these homozygous positives.

In conclusion, there is no indication that carrying a functional \( \text{GSTM1} \) gene is a risk factor for glaucoma in the Swedish population. Previous results from the Estonian study could represent a population specific effect or be the result of sampling bias or chance.
Paper IV: Analysis of rare variants and common haplotypes in the optineurin gene in Swedish glaucoma cases

Background
In 2002, Rezaie et al. reported the identification of a gene in the GLC1E locus. The gene, OPTN (gene product optineurin), was identified in families with mostly NTG. The OPTN gene consists of 16 exons covering ~38 kb of genomic sequence. Translation initiation is located in the fourth exon and alternative splicing of the first three exons produce at least three different isoforms. All isoforms encode a protein consisting of 577 amino acids and a molecular weight of approximately 66 kDa.

In the families included in the study, 16.7% carried sequence variants predicted to cause disease. Most families (seven out of nine) carried the E50K variant. Other mutations found were 691_692insAG and R545Q. M98K, an allele predicted to be risk-associated, was found in 13.6% of familial and sporadic cases of mostly NTG and in 2.1% of controls.

The protein is expressed in TM, nonpigmented ciliary epithelium, retina, optic nerve, ganglion cells and brain, among other tissues. The endogenous protein is located intracellularly to the Golgi apparatus and was detected in samples of aqueous humor from human and several other species. The function of optineurin is unknown, but the protein is implicated in the tumour necrosis factor-α (TNFα) signalling pathway. In addition, the protein may interact with huntingtin, Ras-associated protein RAB8, transcription factor IIA and two unknown kinases. Rezaie et al. speculate that the protein may have a neuroprotective role.

Studies of the OPTN mutation spectra in other populations have so far been inconclusive.

Aung et al. reported an association between M98K and British sporadic NTG patients, but not POAG patients. In addition, they reported E50K as an infrequent cause of sporadic NTG. Alward et al. reported an association between M98K and Japanese sporadic NTG patients, but not Caucasian sporadic NTG. They also reported E50K as responsible for a fraction of Japanese familial NTG. Tang et al. found no associations to Japanese sporadic NTG. Concerning POAG and OPTN, Leung et al. reported patients carrying putative disease-causing mutations in a Chinese cohort of POAG. Whether the patients carrying mutations were high IOP or NTG was not reported. Several other studies failed to show association between OPTN and POAG.

Melki et al. reported an association between the M98K variant and lower initial IOP in French POAG patients, but not in Moroccan POAG patients. They suggest that the M98K variant is a modifier of glaucoma phenotype.

Vittitow et al. performed expression studies which revealed that OPTN is upregulated in human organ cultures grown under pressure (35 mmHg), after
exposure times of 2, 4 and 7 days. There is also an upregulation of *OPTN* after induction by TNFα and by treatment with the corticosteroid dexamethasone⁹⁸. Kamphuis *et al.* studied the expression during shorter time spans (1-24 h) of increased pressure (30 mmHg). This study revealed no increase in *OPTN* expression.° Increased expression of *OPTN* seems to be a delayed response to increased pressure. This may be secondary response, since mutations in the gene is associated mostly to glaucoma with normal pressure.

**Results and discussion**

In this study, we used dHPLC and sequencing to search for and identify sequence variation in the *OPTN* gene. Exons four to six and sixteen were sequenced according to standard protocols, and exons seven to fifteen were analysed using dHPLC. This setup allowed us to analyse the entire coding sequence of all 200 POAG and 200 exfoliative glaucoma patients, and 200 controls in the most efficient manner, by running dHPLC in exons with few reported variants and directly sequence exons containing common variants. In addition to this, we identified and typed nine SNPs distributed throughout the gene, using SNaPshot. We were able to multiplex all nine SNPs in a single SNaPshot reaction.

Our analysis revealed none of the glaucoma causing mutations reported by Rezaie *et al.* Neither was the predisposing 603T>A (M98K) variant found to be predisposing in our Swedish cohorts, but was rather found in 4.5% of the POAG patients, in 5.5% of the exfoliative glaucoma patients, and in 5.1% of the controls. Frequencies of the M98K variant have been found to vary extensively between different populations. Chances are that the M98K variant may be predisposing to NTG in England and parts of Asia, but not in the rest of the world³¹,⁴⁰⁻⁴⁷. Other coding sequence changes found in the present study were 412G>A and 433G>A, but both are silent, and found in patients and controls in equal frequencies.

Haplotypes can be used to reveal associations between disease and gene, even if there is no disruptive mutation in the coding sequence, as is common in complex disease. Nine SNPs were typed in this study and all were used to construct haplotypes. The SNPs analysed were rs478911, 412G>A (T34T), 433G>A (L41L), 458G>A (E50K), 603T>A (M98K), 675C>T (S122F), rs2244380, rs765884, and rs2095387. Of the nine SNPs, two (603T>A and 675C>T) were non-polymorphic in our populations.

Using the Phase program, we constructed haplotypes from the SNPs typed.⁴⁰ Using a computer program to construct haplotypes is somewhat risky, since there is a possibility of assigning the wrong haplotypes to a patient. To test the reliability of the software we analysed haplotypes in 100 nuclear family trios. The program was tested on the genotypes from the offspring, and the result was compared to the one implied form the family structure, without finding any mistakes in the prediction.
There was a surprisingly large number of haplotypes, 23 in all, although four haplotypes were more frequent than the rest. We reconstructed the haplotypes using only high frequency SNPs, but the yield was still a relatively high number of haplotypes.

Initial analyses showed that the frequencies of one haplotype were significantly different between the POAG and control cohorts. This was the only significant result found when comparing either haplotypes or individual SNPs (genotypes or alleles). However, when adjusted for multiple testing, using the Bonferroni correction, the significance disappeared. The 603T>A (M98K) variant was found on a single haplotype in all patients but one. This indicates that the variant is the result of a founder mutation, while the atypical one may have been originated either by a recombinational event between rs765884 and rs2095387, or by a mutation at the latter site.

Summarising the results of this study, we found no association to OPTN in our Swedish cohorts of high IOP glaucoma. This indicates that there is a difference in the molecular genetic mechanisms underlying the different subtypes of glaucoma in this study compared to the subtype in the study by Rezaie et al.
Concluding remarks and future perspectives

Concluding remarks
The works presented in this thesis have resulted in an advancement of the knowledge of the genetic differences between different subgroups and populations of glaucoma. The works included molecular genetic analyses of the genes MYOC, oculomedin, GSTM1 and OPTN.

PAPER I
The MYOC gene has been associated to JOAG and POAG cases of different populations worldwide. We found a frequency of mutations and polymorphisms in our POAG cases comparable with similar studies, confirming the association between POAG and MYOC. Exfoliative glaucoma had not been studied in larger cohorts until now. We found no disease-associated mutations in our exfoliative glaucoma cases, suggesting a fundamentally different genetic origin for this subtype of glaucoma. Mutations found in two families confirm that JOAG may be caused by mutations in this gene.

PAPER II
The oculomedin gene show an increase in expression level in organ cultures under stress. This makes it a candidate gene for glaucoma. We found no genetic evidence of this gene being involved in glaucoma in our cohorts. The expression level difference reported may imply that the presumptive connection to glaucoma is rather connected to the promoter than to the short coding sequence. However, this does not exclude the possibility of causative mutations in the coding sequence.

PAPER III
The GSTM1 gene is deleted in parts of the population. It was reported to be present in a higher frequency in Estonian glaucoma cases, compared to the normal population. We found no differences in the frequency of deleted GSTM1 between our cases and controls, nor in the presence of one or two alleles. The GSTM1 gene may still be involved in the aetiology of glaucoma, but by a subtler mechanism.
PAPER IV
The \textit{OPTN} gene has been associated to glaucoma with normal IOP. We found no genetic evidence of this gene being involved in our cases of glaucoma with increased IOP. Our results indicates molecular genetic differences between normal tension and high tension glaucoma subgroups.

In all, there are just a few per cent of the Swedish POAG patients, with genetic variation associated to disease, in the genes analysed in this study. No genetic variation associated to exfoliative glaucoma was found in any of the genes analysed, indicating that other factors are involved in the aetiology. As can be seen by these studies, genes associated to a disease in one population are by no means certain to be associated to the disease in another population. Whether this is dependent on an inherently heterogeneous genetic basis for glaucoma in different populations or differences in the diagnostic criteria used is presently unknown.

\textbf{Future perspectives}
Glaucoma affects a large part of the world’s population, and is a leading cause of blindness. The only available treatment for glaucoma today is a reduction of the increased IOP present in some forms of glaucoma. This can slow or halt the progression of the loss of the visual field\textsuperscript{111}, but not reverse the damage, making early detection vital.

Molecular genetic tests that can identify people with a high risk of developing glaucoma prior to glaucomatous damage have occurred, requires detailed knowledge of which genes that are involved. In addition, you need to know which subgroup of glaucoma to analyse and possible differences between populations or ethnic groups. Scientists are working towards gaining this knowledge today. At the moment, the heterogeneity of genetic factors and variability in disease associated alleles makes genetic analysis of glaucoma for diagnostic purposes less feasible as in, for example, familial cancer, except in rare glaucoma families.

To further our knowledge about glaucoma, we need to identify additional genes associated to the different subtypes of the disease. This can be done either by using families for linkage analysis or by identifying candidate genes. These genes must then be analysed in different subgroups in several populations, to gain the best knowledge of the genetic spectrum. Additionally, the genes need to be analysed in a sufficiently large number of cases to convincingly identify disease-causing alterations. Possible candidate genes identified already are ApoE\textsuperscript{112,113}, optimed\textsuperscript{114}, TNFa\textsuperscript{115}, OPA1\textsuperscript{116} and atrial natriuretic peptide\textsuperscript{117}.

Although the mechanism through which mutant \textit{MYOC} causes the increase in IOP is unknown, the damage to the retinal ganglion cells is likely caused by this increase. A possible pathway through which increased IOP
damages the optic nerve axons involves excessive nitric oxide generated by inducible nitric oxide synthase (NOS-2). Another possible mechanism is that the retinal ganglion axons are damaged through oxidative stress, due to reduced blood flow in the optic nerve head caused by the increase in pressure.

A complicating factor in molecular genetic studies is the diagnostic criteria used in collecting the disease cohorts. If the criteria are not stringent enough, the cohort will be too heterogeneous to be able to find an association; on the other hand, if the criteria are too stringent the cohort may be too small for useful purposes. In addition, to be able to compare genetic studies from different populations, the disease cohorts need to have been collected using similar diagnostic criteria. To facilitate genetic studies, it would be good if ophthalmologists around the world could agree on subgroup divisions and which diagnostic criteria to use.

Characterisation of implicated chromosomal loci and candidate genes, to understand the molecular basis and aetiology of glaucoma have been a good beginning, but efforts to understand the complex genetics and environmental interactions of the disease have just begun.
Acknowledgements

The work presented in this thesis was conducted mainly at the Department of Genetics and Pathology at Uppsala University. I would like to take this opportunity to express my gratitude to the people who have made these years what they were:

My supervisor, Professor Claes Wadelius, for giving me the opportunity to work in his group and for providing a good scientific environment.

Professor Ulf Pettersson for making the Rudbeck laboratory such a great place to work.

Present and former members of the Group of Ophthalmic Genetics: Benjamin Bakall, Sofie Ingvast, Towa Marknell, Anna Sjöstrand, Petra Sandell (having fun “making babies?”), Fredrik Aspgren, Ola Wallerman, Álvaro Rada (¿Qué pasa?), Mehdi Motallebipour (now the glaucoma project is all yours) and Elin Pless. Girls, I hope it wasn’t something I said… And the undergraduate students, who’s names I have forgotten.

The Group of Clinical Molecular Genetics, Professor Niklas Dahl, Hans Matsson (you’re next, muhahaha), Joakim Klar (UNGE!!!), Larry Mansouri, Malin Larsson (click-click), Miriam Entesarian, Ed Davey, Lena Marklund and Birgit Carlsson, and the people down at the clinic.

All the people at the Department of Genetics and Pathology. I dare not mention anyone, lest I forget someone. But you know who you are. I mean YOU!

Margareta Uvhagen, Jeanette Backman, Elisabeth Sandberg, Maria Hedefalk, Gunilla Åberg and Britt-Marie Carlberg for all the help during the years, and Viktor Persson for computerised reasons.

The personnel at the out-patient clinic at the Department of Ophthalmology, University Hospital, Uppsala, in particular Lill-Inger Larsson and Lidija Tomic.
All the patients with and without glaucoma, who so gracefully agreed to participate in these studies and made this work possible. I sincerely hope my work will in some way benefit you all and contribute to future diagnostics and treatment.

The “Gang”: Jonas (ÖLölÖLölÖL), Thomas, Stefan, Jessica, Ulrika, Aneta (and Emilia), Fredrik, Åsa, Linda, Veronica, Mathias and Kiki. It’s been great fun.

Min familj: mamma Lena, pappa Bosse, mina systrar Ulrika (familjens nästa högutbildade), Sofia (tack för hjälpen med inbjudningarna), Tove (för att du är den du är) och Kaisa (alv, slav eller viking?), mina “plastsyskon” Jimmy (Ja, han missade bollen!), Ivette, Camilla, Sebastian och Sara, Sofias man Fredric och Ulrikas sambo Tommy, och sist men… Ehh, OCH minst, mina systerdöttrar Tindra, Emma och Tuva (ni är ena små gullungar allihop). Tack för att ni finns.

This thesis was supported by grants from the following foundations and companies:

- Swedish Research Council
- 6th of December Foundation
- Kronprinsessan Margareta’s Foundation for Visually Impaired
- Synfrämjandets Research Foundation
- Insite Vision
Summary in Swedish

Molekylärgenetiska studier av gener som predisponerar för glaukom

Många människor får sent i livet nedsatt syn till följd av glaukom, också kallad grön starr. Glaukom är vanligast hos äldre personer där 1-2% av befolkningen över 55 års ålder och ca 4% av befolkningen över 75 års ålder drabbas. Sjukdomen är dock ovanlig hos personer under 40 års ålder.

Glaukom är en sjukdom som angriper synnerven, där skadorna ger en långsam försämring av synfältet med en början i det perifera seendet (Figur 1). Sjukdomsförloppet är i de flesta fall mycket långsamt och medför inget obehag eller smärtor, vilket kan leda till att patienter dröjer med att uppsöka läkare tills en allvarlig synfältsskada har uppkommit. Detta gör att uppskattningsvis hälften av alla glaukomfall i Sverige är odiagnostiserade. De skador som uppkommit på synfältet kan sedan aldrig återfås och kan, om det får fortsätta, i vissa fall leda till blindhet.

Glaukom har en komplex sjukdomsorsak där både gener och miljöfaktorer påverkar. Att det finns genetiska faktorer visas av det faktum att om sjukdomen finns hos en person är risken ca tiodubblad för nära släktingar att också få sjukdomen, jämfört med risken för en person i normalbefolkningen.

De patienter vi undersökt har de former av glaukom som är vanligast i Sverige. Våra patientgrupper består av 200 fall av simplexglaukom, 200 av exfoliationsglaukom samt 200 kontroller där sjukdomen har uteslutsits. För simplexpatienterna skulle inkluderas i studien skulle de ha dokumenterade skador på synfältet, glaukomrelaterade skador på synnerven, och ett högt tryck i ögat. För exfoliationsglaukompatienterna krävdes dessutom pseudo-exfoliativt material i ögat.

I denna avhandling har vi undersökt gener som associerats med glaukom och försökt identifiera mutationer, dvs förändringar, i dessa gener som kan förklara sjukdomen. De gener vi undersökt är MYOC, oculomedin, GSTM1 och OPTN.

**MYOC:** Denna gen har associerats till glaukom i många olika populationer från hela världen. Vi ville se ifall också våra svenska glaukompatienter hade förändringar i denna gen. I våra simplexpatienter hittade vi sjukdomsrionale mutationer i en frekvens som motsvarar den i andra delar av världen, vilket bekräftar denna gens association till simplexglaukom. Exfoliationsg-
Glaukom hade inte tidigare studerats med avseende på denna gen, och vi fann inga sjukdomsrelaterade mutationer i denna patientgrupp, vilket indikerar att i denna sjukdomsrelaterad gen finns andra genetiska komponenter. Vi undersökte även två familjer med årligt glaukom och hittade sjukdomsrelaterade mutationer i båda familjerna.

**Oculomedin**: Denna gen induceras då vävnader från ögat utsätts för stress i form av sträckning, vilket är vanligt vid glaukom. Detta gör den till en gen som kan vara involverad i glaukom, och vi ville undersöka om så var fallet. Vi hittade inga förändringar i denna gen som indikerade att den kunde orsaka glaukom i någon av våra patientgrupper.

**GSTM1**: Denna gen har den egenheten att alla inte har den. Den saknas i delar av befolkningen. Man kan ha antingen två, en eller ingen kopia av genen i sin arvsmassa. I en estnisk studie hittade man en högre frekvens av personer med en eller två kopior av genen hos sina glaukompatienter jämfört med normalbefolkningen. Vi ville se ifall samma skillnad fanns hos våra svenska glaukompatienter. Vi undersökte våra patienter för att se om, och i så fall hur många kopior av genen de hade. Vi såg ingen skillnad i frekvens i antal kopior hos någon av våra patientgrupper jämfört med kontrollerna.

**OPTN**: Denna gen har associerats till patienter med en annan variant av glaukom, där patienterna har ett normalt tryck i ögat. Då våra patienter har ett förhöjt tryck i ögat, ville vi se ifall genen är associerad även till våra patientgrupper. Vi hittade inga förändringar hos någon av våra grupper, vilket indikerar att de olika varianterna av glaukom beror av olika gener.

Totalt sett så var det bara några få procent av våra simplexglaukompatienter som hade sjukdomsrelaterade mutationer i generna vi undersökte. Vi hittade inga mutationer alls i våra exfoliationsglaukompatienter. Detta indikerar att det är stora skillnader mellan olika befolkningsgrupper vad gäller vilka gener som glaukom är beroende av.

Glaukom behandlas idag genom att det förhöjda trycket i ögat sänks. Det kan ske genom medicinering, tex ögondroppar, eller en operation med laser eller konventionell kirurgi. Målet med dessa och följande studier är att utveckla metoder för att kunna upptäcka personer med hög genetisk risk för att utveckla glaukom, så att de kan behandlas innan skador på synnerven, och därmed synfältet uppkommit, och även att ta reda på mer om de bakomliggande mekanismerna för sjukdomen så att nya läkemedel kan utvecklas.
References

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to October, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Medicine”.)