Aims and Objectives
Environmental and lifestyle factors, aging process, diet and genetic composition are all known to be involved in the causation of many common diseases. Glaucoma is one such multifactorial complex neurodegenerative disease which is the second largest cause of irreversible blindness (after cataract) affecting 80 million people worldwide. It is characterized by the progressive loss of retinal ganglion cells (RGC) and their axons, and is clinically recognized by progressive excavation of the optic nerve head and resultant visual field loss (Mozaffarieh et al., 2008). Increased intraocular pressure (IOP) is a major risk factor of primary open angle glaucoma. However, more than 30% of the glaucoma patients suffer from optic nerve damage without elevated IOP (normal tension glaucoma). It is still a matter of debate whether apoptosis necrosis is the mechanism of RGC death in glaucoma.

Literature review shows, Hyaluronan (HA) may play an important role in the glaucoma pathogenesis that has already been discussed in the earlier section. Briefly, the molecule acts as a ‘lockgate’ component in the trabecular meshwork that controls aqueous humor flow and protects the anterior chamber from shrinkage. High viscosity and ROS scavenging potential have turned hyaluronan into a very important component in the field of glaucoma research. During glaucoma along with other GAGs, HA is deposited on the trabecular wall reducing the flowchannel diameter. It is thought that this process possibly disturbs the natural aqueous flow balance and thereby influences high-pressure on the retinal ganglion cells and optic nerve. A change in its normal distribution pattern has been reported in several other parts of the eye in this disease.

Although there is a high probability of the involvement of HA, its synthesis, function and degradation, very little work has been done on the hyaluronan metabolism as a whole in this eye disease. Even an elastoviscous solution of high molecular weight hyaluronan, commercially known as Hylan is used frequently in ophthalmic viscosurgery and for the treatment of painful dry eye syndrome acting as a protective agent for discomfort caused by desiccation of corneal surface or by irritations due to environmental factors. Till date, only a few number of genes (like CD44 and Versican) related to this unique metabolic pathway have been studied. Therefore in the present study, our aim was to
explore the importance of hyaluronan metabolic genes and their expression regulation and functional involvement in the process of glaucoma neurodegeneration.

**Selection of the hyaluronan metabolic genes:**

As mentioned earlier, hyaluronan is synthesized at the inner surface of the plasma membrane by three related isoenzymes, hyaluronan synthases (HAS1, HAS2, HAS3) which is located on 19q13.4 chromosome. The expression levels of each HAS gene and protein are regulated by several cytokines and growth factors, such as platelet derived growth factor BB (PDGF-BB) and transforming growth factor β (TGF-β). In glaucomatous eyes, it is reported that the TGF-β levels get elevated in aqueous humour. Usui et al. (2003) showed that in the cultured bovine trabecular meshwork cells, among all the hyaluronan synthase genes, HAS2 gets upregulated maximum in response to TGF-β and PDGF-BB both in mRNA and protein level. HAS2, principally responsible to synthesize high molecular weight hyaluronan may have significant importance during developmental process of eye (Camenisch et al., 2000). Therefore, for our present study we have selected HAS2 gene.

Hyaluronan performs diverse biological functions interacting with special proteins called hyaladherins. Our laboratory is working on one such protein, HABP1/C1QBP/p32 which is involved in several HA mediated activities, eg. Cell adhesion, tumorigenesis and apoptosis. It's present on the gene 17p13.3. As its name suggests, it also binds to the globular head of the complement activation factor C1q. Recently, HABP1/C1QBP/p32 has been found interacting with Human Forkhead Box C1 (FOXC1) that is principally responsible for Axenfeld Rieger malformations, often leading to glaucoma (Huang et al., 2008). While HABP1/C1QBP/p32 is predominantly cytoplasmic, the portion of this protein inside the nucleus co-localizes with FOXC1. It helps in regulation of FOXC1-mediated transcription activation. The study showed a mutation (F112S) in the FOXC1 hindered its interaction with C1QBP that might be responsible for causing eye disease. Complement activation in retina is also evident along with IOP elevation having significant implications in the pathophysiology of glaucoma. Stevens et al. (2007) showed C1q became upregulated and synaptically
relocalised in the adult retina early in the disease in a mouse model of glaucoma. The study by Kuehn et al (2006) supported this fact. Like Myocilin, another key protein responsible for glaucoma, C1QBP/HABP1/P32 also bears a C1q binding domain as already mentioned earlier, thus it may have a potential role in the pathogenesis of glaucoma. So this gene was selected for our present study.

Hyaluronan is degraded by Hyaluronidases which is encoded by several genes. HYAL1, HYAL2 and HYAL3 lie close to each other on chromosome 3p21.3. Several groups have indicated possible involvement of Hyaluronidase in Glaucoma, but no study has yet reported involvement of any one of the above mentioned genes in neurodegenerative diseases. Benozzi et al., (2002) suggested that on the application of brimonidine, a highly selective α2-adrenoreceptor agonist, the hyaluronidase activity gets stimulated that clears GAGs with subsequent increase in aqueous outflow. Hyaluronidase is applied along with plasmin for the induction of posterior vitreous detachment. Apart from involvement in glycosaminoglycans catabolism, the three genes have some definite functions. HYAL1 is involved in cell migration during embryonic development and also involved in the angiogenic and invasive front of tumors. HYAL2 especially lysosomal, specifically hydrolyzes high molecular weight hyaluronan. HYAL3 is secreted in testis and is involved in fertilization. It may also have potential role in stem cell regulation. Several studies indicate that various neurodegenerative diseases have association with azoospermia and infertility. Kuo et al (2005) showed, during Pantothenate Kinase- associated neurodegeneration (PKAN, formerly known as Hallervorden-Spatz syndrome), resulting from an inherited defect in coenzyme A biosynthesis, retinal degeneration and decline in progressive photoreceptor occur. Additionally, the homozygous male mutants become infertile due to azoospermia and the spermiogenesis gets arrested with complete absence of elongated and mature spermatids. Harrison SA (2008) reported that both infertility and neurodegeneration are associated with insulin resistance. Mutation in ATM gene responsible for ataxia telangiectasia causes infertility (McKinnon PJ, 2001). As any mutation in HYAL3 may cause infertility and azoospermia, its apt to question its role in neurodegenerative disorder and hence, this gene was for the present study.
Thus, in order to elucidate the role of hyaluronan metabolism in glaucomatous condition, we propose to study the following candidate genes HAS2, HYAL3 and HABP1.

**Strategy of disease association study:**
Broadly we adopted two approaches to find out the involvement of the selected genes in glaucoma pathway: (a) Genetic approach: Case-control study using Single Nucleotide Polymorphism (SNP) as genetic marker (b) Functional approach: *in vitro* expression analysis of the candidate genes and checking its correlation with the genetic data

(a) **Genetic approach to dissect the involvement of genes**
The ability to genetically map complex disorders has been facilitated by technological improvement in identifying and genotyping polymorphic DNA markers. Single Nucleotide Polymorphisms (SNPs) are most commonly used genetic marker as they are found abundantly (accounting around 90% of the total human genetic variation) in the human genome. They are very useful to track the disease causing genes. These variants account for the vast majority of polymorphism responsible for human diseases. The variation occurs in both coding and intervening regions at a frequency of approximately 1 per 1000 base pairs. Already, the initial map of human genome sequence variation presented by the International SNP Map Working Group ([www.wi.mit.edu](http://www.wi.mit.edu)), has identified around 1.42 million SNPs throughout the genome most of which are located in the non-coding regions. Construction of this genome wide SNP map is an important benchmark in characterizing and correlating genes with complex traits (Rothberg, 2001).

We were interested to observe the nature of these genes specifically in Indian population. During the last few decades, the prevalence of complex genetic diseases like coronary artery disease, diabetes, glaucoma and metabolic syndrome has risen in India considerably and is now greater than in most other populations. There is an extensive social, cultural, linguistic and biological diversity in India. As this country represents more than one sixth of the total world
population, scanning genomic variation in its different populations and sub-populations may provide unique and valuable information related to genetic complexity hidden behind the manifestation of various common and complex disorders. Furthermore, the recent outcome of the phase I data of the Indian Genome Variation Consortium (IGVC) in ethnically well-defined 43 different populations representing the entire spectrum of diversity within the tropical subcontinent has made us very much curious to check the status of our selected genes in the Indian scenario.

The genetic study was broadly divided in two parts. Prior to case-control study, we were curious to see how the SNPs of the selected genes behave in normal population. The case-control study is expected to be the most powerful association study for detecting modest disease risk alleles in common diseases and its basic design is both straightforward and logical. It searches for allele frequency differences between trait barriers (glaucoma) and non-carriers (controls).

(b) Functional assay to explore the glaucomatous pathway:
Apart from the excavation of the role of the selected hyaluronan metabolic genes, in the present study we were interested to explore the neurodegenerative pathway that leads to glaucoma under oxidative stress and how the genes of our interests behave in this process. It is already mentioned that during glaucoma, especially in the high tension category, the trabecular meshwork is actively involved as it controls the aqueous humor outflow in the eye. It's also well established that the retinal ganglion cell death is a hallmark of glaucoma. Understanding their physiological importance in glaucomatous neurodegeneration, we chose Human trabecular meshwork (HTM) and rat retinal ganglion cells (RGC-5) for our study.

In the following chapters, these two cell lines will be discussed more elaborately. Briefly, the retinal ganglion cell is the retinal layer on the inner surface of retina. It receives the visual information from photoreceptors through the bipolar and amacrine cells. RGC-5 is a retinal ganglion cell line transformed in rat with the
ψc2 E1A virus (Krishnamoorthy RR et al., 2001). HTM cell line was originally collected from a normal individual.

Various experimental reports suggest that reactive oxygen species (ROS) induces glaucoma. It is hypothesized that due to high IOP, axonal transport is blocked within the optic nerve head that eventually causes obstruction in the retrograde neurotrophin transport. It leads to RGC death by the process of apoptosis that finally leads to vision loss (Charles et al., 2005). Significantly high oxidative DNA damage occurs in the ocular epithelium regulating aqueous humor outflow, i.e., the trabecular meshwork (TM) in the glaucoma patients. It has been revealed that aqueous humor outflow is disturbed due to hydrogen peroxide (an ROS) induction. Also, the antioxidant mechanisms involving superoxide dismutase–catalase and glutathione pathways alter along with increased IOP due to ROS induction (Izzotti et al., 2006).

**Objectives of the study:**

In order to find out the involvement of three hyaluronan related genes, namely *HABP1, HAS2* and *HYAL3* in the glaucoma pathogenesis in Indian population, the present study broadly consists of two parts. The first part includes the genetic analysis while the next one validates and tries to correlate the genetic observation functionally.

**A. Genetic analysis:**

The allele frequency and the haplotype frequency of the selected SNPs of the three genes (*HAS2, HABP1* and *HYAL3*) would be analysed in 24 different normal Indian populations. Comparative allele and haplotype frequency study of the selected SNPs of the same genes would be done between the glaucoma patients and unrelated unaffected samples in an Indian population to check whether the SNPs have any genetic association with glaucoma (case-control association study).

**B. Functional analysis:**

The glaucomatous condition would be simulated *in vitro* in the two cell lines (RGC-5 from rat and HTM from human) by different conditions. On confirmation
of the simulated glaucomatous state, the cellular behavior under the given experimental conditions would be studied. The expression, localization and different functional activities of the three proteins (HAS2, HYAL3 and HABP1) along with other proteins linked to glaucoma would be analyzed. Further, transient transfection as means of overexpression would be used to study the functional significance of any of the three genes in in vitro glaucomatous condition. Finally, the in vitro expression studies would be validated in the clinical samples of aqueous humor from glaucoma patients and normal individuals.
Chapter I

Involvement of Hyaluronan metabolic genes in Glaucoma: A genetic approach