Summary
Glaucoma, a multifactorial complex neurodegenerative disease is the second largest cause next to cataract in the whole world, for irreversible blindness. 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).

As in most neurodegenerative diseases, the cellular pathophysiology of glaucoma is poorly understood reflecting its complex multifactorial aetiology. Being a multifactorial disease, several pathways are involved in this disease. Glutamate toxicity, serum starvation, vascular dysregulation, mechanical stress etc., are some important etiologies in this neurodegeneration. Among all of them, oxidative stress plays a common role in retinal ganglion cell death during glaucoma (Munemasa et al., 2008). The cellular signalling of this degeneration depends on interaction with matrix proteins and cell surface receptors. The complex network of ECM proteins helps in modulation of neuronal communication, regulates plastic changes and protects neurons and synapses against damage by regulating synaptic differentiation and function by interacting with their cell surface receptors, interacting with ion channels and transmitter receptors, hypothesing that the ECM may be involved in neurodegenerative diseases (Bonneh-Barkay et al., 2008). Being an important component of the extracellular matrix, Hyaluronan (HA) immobilizes large amount of water and ions and affects extracellular compartmentalization of macromolecules by way of steric exclusion. It is known to be implicated in regulation of cell migration, proliferation, adhesion, and differentiation. It plays an important role in regulating physiological balance in the eye by maintaining intracellular viscosity. During glaucoma alongwith other GAGs, HA is deposited on the trabecular wall, reduces the flowchannel diameter. It is thought, possibly by this process it disturbs the natural aqueous flow balance and thereby influences high-pressure on the retinal ganglion cells and optic nerve. Increased intraocular pressure (IOP) is a major risk factor of primary open angle glaucoma.

Hyaluronan is synthesized at the inner surface of the plasma membrane by three related isoenzymes, hyaluronan synthases (HAS1, HAS2, HAS3), degraded by the enzyme Hyaluronidase and its function is regulated by a family of protein.
Being the second most important glycosaminoglycan (GAG) of the trabecular extracellular matrix in the anterior chamber of the eye, its involvement in the glaucoma pathogenesis is well reported. The modulation in the natural balance of hyaluronan in the trabecular meshwork may play a key role in inducing glaucoma (Knepper et al., 2005; Navajas et al., 2005).

Although increased intraocular pressure (IOP) due to the imbalance in hydrolic pressure in interior and posterior chamber of eye is a major risk factor for POAG, other concomitant factors affecting the eye leading to cell death plays an important role in this neurodegeneration. Imbalance between the production and removal of reactive oxygen species (ROS) caused by oxidative stress may play a major role in the pathogenesis of this disease. It is speculated to be a key player in inducing and maintaining degeneration of the optic nerve and the retinal ganglion cells, which determine the progressive and irreversible deficit of the visual function. This neuronal loss in glaucoma is linked with the cellular signalling through programmed cell death termed as apoptosis. Hyaluronan is already reported to be involved in the process of oxidative stress. It itself acts as a scavenger of ROS and breaks down into low molecular weight oligosaccharides.

Keeping in mind all the given observations, the present study focuses on the prospective role of this important glycosaminoglycan with its important metabolic genes like synthase (HAS2), Hyaladherin (HABP1) as well as degrading enzyme (HYAL3).

Complex diseases like glaucoma involving such multiple manifestations are studied using genetically isolated populations and mapping underlying genes related to them. In the determination of such multifunctional complex diseases in various populations as a consequence of differences in allele frequencies arising mainly due to natural selection, genetic drift is a major problem faced. So to assess the nature and extent of population stratification in contemporary endogamous populations, candidate disease genes has been targeted.
In order to find out the involvement of the 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.

**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, after confirmation of glaucomatous protein, 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) alongwith other proteins linked to glaucoma would be analyzed. Further, transient transfection as means of overexpression would be used to study the functional significance of one any of the candidate genes would be determined in *in vitro* glaucomatous condition. Finally, the *in vitro* expression studies would be validated in the clinical samples of aqueous humor samples from glaucoma patients and normal individuals.

We first observed a significant allelic association (rs6651224; \( p = 0.031 \)) at the second intron and a genotypic association (rs1057308; \( p = 0.02756 \)) for the recessive model at the 5' UTR of HAS2 with only high tension group of glaucoma. The C>G allelic change of rs6651224 has a protective role (0.083 in case and 0.16 in controls) in cases. The associated GG genotype of rs1057308 is a risk genotype. HABP1 and HYAL3 did not have any allelic or genotypic association.
with the POAG patients, but their haplotypes are significantly linked to the diseased condition. Both of their haplotypes were found associated with the HTG and NTG groups and all are risk haplotypes. The significant allelic and the genotypic association indicates, HAS2 may be involved only in the high tension glaucoma. In normal tension glaucoma it doesn’t play any potential role. In spite of no allelic and genotypic association, significant haplotypic association indicates HYAL3 and HABP1 can modulate hyaluronan level in both high tension and normal tension glaucoma.

From this in silico analysis, it can be concluded that these hyaluronan metabolic genes are probably involved in glaucoma pathogenesis in two different routes. In both, high tension and low tension glaucoma, hyaluronan binding protein (hyaladherin) HABP1 may act as an interacting partner. In high tension glaucoma, it interacts with the hyaluronan synthesizing HAS2 while during normal tension glaucoma, its interaction occurs with hyaluronan degrading HYAL3.

To validate the genetic association data functionally, we used simulated glaucoma condition in the cultured cell line. We used the transformed rat retinal ganglion cell line RGC-5 resembling the characteristic phenotypes of the retinal ganglion cells (Krishnamoorthy et al., 2001) for our study.

In the present section, we first tried to simulate the glaucomatous condition in RGC-5 cell line by oxidative stress. When the cells were starved of serum for 72 hours, ROS was generated (about 4.6 fold increase) while under 500uM H2O2 treatment ROS was increased about 4.7 fold. We checked the expression of different proteins related to glaucoma to confirm the glaucomatous condition in the RGC-5 cell line. Nuclear translocation of GAPDH showed the cell was under stress at 72 h serum deprivation. Expression of Bax and its nuclear translocation confirmed that apoptosis occurred under both the sources of oxidative stress. Expression of Gamma Synuclein confirmed the cell line was RGC-5. Myocilin’s colocalization with gamma synuclein in the nuclear periphery of the cell confirmed that the glaucomatous environment was developed in the RGC-5 cell under respective oxidative stress. Subcellular translocation of MMP9 also confirmed the development of glaucoma like condition hinting towards IOP elevation, as an
increase in MMP9 activity is already reported at high IOP. The observation from all expression studies confirmed the generation of glaucomatous condition in RGC-5 under oxidative stress (H$_2$O$_2$ (500uM) treatment) and serum deprivation (72h).

Though the state of oxidative stress is created in the RGC-5 cells using SFM or H$_2$O$_2$ treatment, the resultant change in cellular morphology in both the cases is strikingly different. Conspicuous black spots are observed in nucleus of cells undergoing SFM treatment. Prominent chromosomal condensation with an interesting depression on the cell surface is observed in the electron microscopy data on SFM treatment. This could be attributed to nuclear remodeling under nutrient deprivation stimulation. No such change is observed on H$_2$O$_2$ treatment. The phenomenon of exocytosis is observed in cells treated with H$_2$O$_2$. But there’s a significant alteration in the nuclear/cytoplasmic ratio of the cells under oxidative stress condition generated in both the cases. The variation in cellular morphological aberration under different sources of oxidative stress suggests the cell’s ability to undergo different signalling pathway respectively.

With the availability of simulated *in vitro* glaucomatous condition in RGC-5 cells, we continued our studies to validate the involvement of the candidate genes in the development of glaucoma.

We observed that *in vitro* simulated glaucomatous condition in RGC-5 hyaluronan changed its localization. It shifted to nucleus predominantly, from its natural diffused cytosolic and nuclear peripheral localization. The glaucomatous condition did not have any significant effect on the expression and localization of HABP1. But when HABP1 is exogenously added in RGC-5, it induces anchorage independent cell survivability, but did not have any effect on cell migration. When HABP1 was transiently overexpressed, it induced ROS (~2.5 fold) without altering the cellular morphology. Under transient overexpression of HABP1 in RGC-5, there’s an upregulation in Myocilin expression though there’s no change in its localization pattern. Hyaluronan supplementation restored RGC-5 cellular growth whereas hyaluronidase inhibited cell survivability. In glaucoma patients we observed downregulation of HAS2, but in *in vitro* simulated glaucomatous
condition its expression was found higher both in HTM and RGC-5 cells under 500uM H₂O₂ treatment. Under dexamethasone and high glucose treatment downregulation of HAS2 was observed. Interestingly, in H₂O₂ treated condition we could see HAS2 colocalizing with myocilin in the nuclear periphery.

In conclusion, it's interesting to note that the genetic analysis puts forward the association between the hyaluronan metabolic genes HAS2, HABP1 and HYAL3 in the neurodegenerative disease, glaucoma. The functional analysis of the target proteins in in vitro simulated glaucomatous conditions are a reflection of the genetic analysis. Studies in RGC-5 and HTM cell line corroborate the possible role of the candidate genes in the progression of the disease. This opens up new vista in the field of glaucoma pathogenesis.