David Tvildiani Medical University

Nino Kobakhidze

Exfoliation Syndrome in Georgian Patients Undergoing Cataract Surgery

Thesis of Dissertation for the PhD Academic Degree

in Medicine

Scientific Advisors:

Sergo Tabagari, MD, PhD, Prof. David Tvildiani Medical University

George Chichua, MD, PhD, Prof. New Vision University

Tin Aung, MD, PhD, Prof.Singapore Eye Research Institute

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Publications

1. N.Kobakhidze, G. Chichua, C. Khor, T. Aung - Genetic Markers of Exfoliation Syndrome in Georgian Population - 36th Congress of ESCRS, 10-14 September, 2016, Copenhagen, Denmark - Poster
2. N.Kobakhidze, S. Tabagari, G Chichua - Genetic markers of exfoliation syndrome in Georgian population - 13th International Postrgraduate Conference, Hradec Kralove, Czech Republic, Nov 24-25, 2016
3. Aung T, Ozaki M, Lee MC et al. - Genetic association study of exfoliation syndrome identifies a protective rare variant at LOXL1 and five new susceptibility loci. *Nat Genet*. 2017;49(7):993-1004. doi:10.1038/ng.3875
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5. N.Kobakhidze, S. Tabagari, G Chichua - LOXL1 Gene Variants in Association with Exfoliation Syndrome in Georgian Population Georgian Medical News , N1 (286) Jan 2019
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Oral Presentations

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List of Abbreviations

XFS – Exfoliation Syndrome

XFG – Exfoliation Glaucoma

PEX – Pseudoexfoliation Syndrome

ROS - Reactive Oxygen Species

MMP – Matrix Metalloproteinase

CAD – Coronary Artery Disease

IHD - Ischemic Heart Disease

CTR – Capsular Tension Ring

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Introduction

Relevance of the Problem

Exfoliation Syndrome (XFS) is an age-related disorder affecting millions of people worldwide. The exact prevalence of this condition in Georgia is not known but ophthalmologists and especially anterior segment surgeons agree that it is extremely common among cataract patients. XFS is a major cause of most of the technical issues leading to most severe complications encountered during phacoemulsification.

XFS was first described by Lindberg in 1917 in his PhD thesis and it was published later in 1989[1]. Following the first publication of this syndrome, numerous clinical and morphological studies have been performed to understand the different ocular aspects of the syndrome during past decades. At first the disease was named as Pseudoexfoliation Syndrome (PEX), though, nowadays, the two terms are used interchangeably.

Exfoliation syndrome is characterised by the production and progressive deposition of abnormal fibrillary extracellular material in ocular tissues. XFS manifests in all parts of the anterior segment of the eye, in conjunctiva and in orbital structures. XFS is a chronic disorder, which does not show any symptoms. It is characterised by slow development and the presence of subclinical signs. These signs usually are not difficult to be detected clinically. There are ongoing research efforts to understand this syndrome. However, the exact aetiology and pathogenesis of XFS is yet not known. The typical finding of XFS seen by slit-lamp examination of the eye, is the existence of a grey, granular material on the anterior lens surface and along the pupillary border. Interestingly, the deposition of a such fibrillary material has been described in skin and connective tissues as well[2,3]. Nowadays, XFS is already considered as systemic disorder, however it's clinical relevance is still almost exclusively studied with regard to the eye. XFS deposits are found in lung, kidney, liver, gall bladder and in meninges. The

literature about the association of XFS with cerebrovascular and cardiovascular diseases is controversial[4]. According to the study of Gökce et al. XFS is characterised by a tendency of increased thickness of carotid intima and increased renal artery resistance[5]. Based on the meta-analysis results of the existing literature, it was concluded that XFS is also associated with elevated risk of vascular diseases, such as aortic aneurisms, coronary heart diseases and cerebrovascular diseases[6].

The diagnosis of XFS is extremely important in clinical ophthalmology, because it has been shown that XFS represents the major risk factor for complications during cataract surgery[7]. There are many reasons which can cause intraoperative and postoperative complications of phacoemulsification in XFS patients. These reasons include: zonular dialysis; capsular rupture; weakened capsule and zonular apparatus, which in turn is resulted from the progressive proteolytic degradation of the suspensory ligament; nuclear luxation; vitreous loss; decentration or dislocation of the intraocular lens with time. Additional complications during surgery, may also be encountered as the consequence of poor and/or inadequate pupil dilation. In XFS patients postoperative anterior chamber inflammation and fibrinous reaction is also frequent, which is caused by weakened blood-aqueous barrier[8]. In eyes affected with XFS, there are frequent corneal endothelial morphological and functional alterations, which makes them more susceptible to surgical trauma. Such a trauma may finally result in transitory, as well as permanent corneal decompensation[9].

Nowadays, XFS is considered as the most common identifiable cause of glaucoma. It has been shown, that glaucoma occurs more commonly in the eyes with XFS than in the eyes without XFS[10]. In some countries XFS represents the major cause of glaucoma[11]. Glaucoma itself is a group of disorders, characterized by the progressive degeneration of the optic nerve. This causes irreversible visual impairment and finally blindness. Glaucoma is the number one cause of irreversible blindness world-wide. Major risk factor of the glaucoma is elevated intraocular pressure. Exfoliation glaucoma

(XFG) is now considered as secondary open-angle glaucoma. Fibrillary material is deposited the trabecular meshwork causing elevation of intraocular pressure, optic nerve degeneration, and glaucoma[12]. 25% of all open angle glaucoma cases are XFG[13]. XFG represents the glaucoma with more aggressive clinical course, compared to other glaucoma types and is associated with significantly increased risk of blindness and vision loss compared to primary open-angle glaucoma[14]. These patients are more resistant to medical treatment and need earlier laser and surgical interventions to control the disease. With regard to angle closure mechanism, it has been suggested to be related to zonular weakness and resulted anterior displacement of the iris[15].

Previously, it has been noted that XFS patients with ocular hypertension progress to glaucoma twice as often as patients with ocular hypertension without XFS. The results were consistent even in cases with where matched for baseline gender, age and intraocular pressure, age[14]. In addition, Moghimi et al. recently discovered that the lamina cribrosa is significantly thinner in the eyes with XFS in non-glaucomatous patients, compared to controls[15]. The authors also found that nonsignificant difference in inferior and superior lamina cribrosa depth in XFS patients compared to controls. However, the importance of these findings in the eyes of XFS patients with regard to the disease progression in glaucoma is not yet determined[16].

Ophthalmologists agree that XFS is extremely common in Georgia, though its exact prevalence is not known. It is a major cause of severe complications in cataract surgery and the reason of secondary glaucoma, a very fast progressing and blinding type of the disease. The mechanisms of disease development are not known to date, though many studies point to genetic factors. Of special interest is the fact that not all XFS patients develop glaucoma, meaning that the underlying factors may be different. Given that Georgians have their own unique genetic ancestry, it is important to evaluate the relevance of the scientific data to our population.

Scientific Novelty of the Study

This is the first attempt to characterize XFS in Georgian patients undergoing cataract surgery. We evaluate its burden on phacoemulsification and its outcomes. We also evaluate these patients for their risks of vascular comorbidities in order to compare with very inconsistent literature on this topic. Most importantly, for the first time in Georgia, we characterize the genetics of XFS for better understanding mechanisms of its development.

The Aim of the Study

The aim of this work was to study the burden of XFS in cataract surgery, to identify the epidemiology and genetic markers of XFS and XFG among Georgian patients, as well as to define the association of the syndrome with systemic vascular conditions.

The Objectives of the Study

1. To study the prevalence of XFS in Georgian patients undergoing cataract surgery.
2. To study the prevalence of XFG in Georgian patients undergoing cataract surgery.
3. To identify systemic vascular conditions associated with XFS.
4. To study the association of polymorphisms of LOXL1, CACNA1A, POMP, SEMA6A, TMEM136, RBMS3 and AGPAT1 with XFS in patients undergoing cataract surgery.
5. To study the association of polymorphisms of LOXL1, CACNA1A, POMP, SEMA6A, TMEM136, RBMS3 and AGPAT1 with XFG in patients undergoing cataract surgery.

Main Points for Defence

1. The prevalence of XFS and XFG in Georgian patients undergoing cataract surgery.
2. The prevalence of vascular comorbidities in patients with XFS undergoing cataract surgery.
3. Genetic markers of XFS in Georgian patients undergoing cataract surgery.

Practical Value of the Study

Given the high prevalence of XFS in Georgia, cataract surgeons often have to deal with serious issues during phacoemulsification. Often times XFS is overlooked by general ophthalmologists and many patients seek for surgery later in the course of disease. The letter factor is also the cause of under-treatment of many glaucoma patients, later referral to specialized eye care centres often results in worse outcomes. The presence of XFS is not considered as reliable reason for faster approval of surgery by Georgian health care and insurance systems, again leading to belated interventions. This study is an important first step forward in improving our results in cataract surgery and glaucoma care.

Chapter 1. Literature Review

1.1 Epidemiology of Exfoliation Syndrome

The exact epidemiology of XFS is not very well studied and the available information on the incidence of XFS is limited[17]. Generally, it has been estimated that there are 60-70 million individuals affected with this disease[18–20]. According to the recent review from Konstas and colleagues[16] the difficulty of the defining of the exact epidemiology of XFS is related to the difficulty of clinical diagnosis of XFS[21] as well as to the use of the different diagnostic methods[20]. Nevertheless, in some study populations the incidence of XFS is estimated as 0 to 39% of the population[22]. The underdiagnoses is thought to be the cause of the low prevalence of XFS in many countries. In all studied reports, it has been shown that the prevalence of XFS is increased with age, whilst the influence of the gender to the development of the disease varies much more[23]. Studies from Hiller et al.,[22] and Astrom et al.,[24] report that XFS is less prevalent in men compared to women, whilst the investigations from others report the opposite[24,25] and some studies do not report the gender difference at all[26,27]. The summary of different epidemiological studies and the prevalence of XFS in various populations are given in Table 1.

Table 1. Prevalence of exfoliation syndrome in various studies. *Adapted from Arnarsson et al., Acta Ophtalmologica, 2009*

Region	50-69 years %	>70 years %	Author
Eskimoes	0	0	Forsius (1988)
Toulon, France		3.6	Colin et al. (1988)
Denmark		4.8	Backhaus et al. (1966)
Hisayama, Japan	2.2	5.9	Miyzaki (2005)
India	4.5	11.9	Arvind et al. (2003)
Peru	0.8	14.3	Forsius (1988)
India	8.2	20.1	Krishnadas et al. (2003)
Brest, France		20.6	Colin et al. (1988)
Turkey	4.6	22.9	Yalaz et al. (1992)
Iceland	5.6	22.6	Arnarsson et al. (2007)
Finland	10	25.3	Forsius (1988)
Epirus, Greece	15.6	29.7	Stedaniotou (1990)
Iceland	11	31.5	Forsius (1988)
Crete, Greece	7.2	39.1	Kozobolis et al. (1997)

It has been considered that the environmental and geographical factors play an important role in the prevalence of XFS and it is characterized with higher specificity in some geographic areas and even varies within the same country. For example, the study in the middle Norway, surveying three population cohorts from separate areas with the same diagnostic method, showed the prevalence of the disease 10.2%, 21.0% and 19.9% of the population[28]. As early as in 1960 XFS was considered as 'Scandinavian' disease, because there were highest reports from Scandinavian countries. However,

more modern studies show that XFS is more or less spread all over the world. There are conflicting results with regards to geographical distribution of the disease, which might indicate that these differences are caused by differences in sampling and diagnostic methods, rather than true geographical differences[29]. There are only single studies performed in some geographical areas (Table 2) and true randomized studies are lacking. However, it has no doubt that XFS is the common cause of the glaucoma in many countries. The XFS material which is found during the examination by slit-lamp represents the pre-requisite for the diagnosis of XFG. The prevalence of open-angle glaucoma, including XFG is higher in northern Europe, including, Iceland, Finland, Middle Sweden and Middle Norway[29]. There are only large number frequency studies, so called category 1 and 2 reports performed so far, whilst more informative incidence and/or prevalence surveys, named categories 3 and 4 studies are lacking. Category 1 and 2 studies has been previously described by A. Ringvold[30] in his review, according to which collected data can be grouped into several categories: (1) data, which is collected from voluntary patients, attending to ophthalmology checkup for some reason; (2) Non-random screening studies of larger groups; (3) prevalence studies and (5) only one incidence study[30]. In conclusion, there is no doubt that XFS occurs worldwide. However, more category 3 and 4 studies are necessary to understand it's true prevalence in different regions.

There are still some notions about environmental and geographical factors implicated in the development of XFS and XFG. In 1988 survey from Norway found a significantly higher coincidence of the disease amongst married couples, which indicates that shared environmental factors might play a role[31]. In US the latitude effect has been observed. It has been shown that the risk of the development of XFS/XFG was higher in Northern parts of US with the hazard ratio (HR) 2.14 (95% CI: 1.94-2.35), compared to Southern parts (HR=0.83, 95%CI:0.75-0.93). As for environmental factors climatic factors seem to be most closely related to the development of XFS and XFG.

Particularly, people who spend most of the times outdoors are at increased risk of the development of the disease[32]. Stein and colleagues examined the following factors in relation to XFS and XFG: mean annual number of sunny days, mean annual snowfall and rainfall and snowfall, mean temperature in summer and in winter[33]. They found that more sunny days were associated with the increased risk of XFS/XFG[33]. Another environmental risk factor for XFS is the colder and ambient temperature[33]. Overall, the eye can be viewed as an organ which is extremely sensitive to UV irradiation and cold exposure. Another thing to consider is the dietary habits which vary in different geographical locations. For example, it is known that coffee consumption is higher in Scandinavian countries, where it estimates about 10 cups per day per person (chartsbin.com). Other countries include Greece and Turkey. It is known that XFS is characterized with high prevalence in these countries.

In addition, it is known that coffee consumption significantly increases homocysteine levels and on the other hand the levels of homocysteine are increased in patients with XFS compared to controls[34], which will be further discussed in XFS biochemistry section below. The Reykjavik study reported that people who consumed more fruits and vegetables had significantly lower risk of XFS[35]. Worldwide meta-analysis indicates that low folate levels and high serum homocysteine levels are associated with the increased risk of XFS/XFG[36]. Overall, the data indicate that certain environmental and geographic factors might play a role in the development of XFS and XFG.

1.2 Morphology of Exfoliation Material

The morphology of exfoliation material has been studied by standard diagnostic hematoxylin and eosin (H&E) stained specimens, by electron microscopy, as well as by immunohistochemical method. Evaluation of H&E stained sections reveal, that

exfoliation material is eosinophilic in nature. In addition, the staining with periodic acid-Schiff stain is positive, which is indicative of the deposition of carbohydrates in exfoliation material[36]. Electron-dense fibrils, which are randomly oriented and embedded in an amorphous ground substance is identified by electron microscopy. These are called exfoliation matrix and exfoliation fibers respectively. Exfoliation fibers are of 22-45 nm thick and they are characteristically cross-banded. The periodicity of cross bands is 50-55 nm. These fibers are different from collagen. The subunits of these fibers are 8 to 10nm in size and are more closely associated to elastic microfibrils[37]. By immunohistochemistry, it has been shown that many basement membrane components are presented in exfoliation material, including fibronectin, laminin and nidrogen[38]. In addition, carbohydrate epitope HNK-1 can be found in exfoliation material. Other markers detected in exfoliation material are fibrillin (major component of elastic fibers), vironectin, microfibril-associated glyco- protein-1, and latent TGF β-binding proteins[39].

Exfoliation material is differentially distributed in 4 different zones in anterior *lens* surface. In central zone, which corresponds pupil, light microscopy examination reveals short and stubby, inconspicuous deposits, while lens capsule appears to remain normal morphologically. In an intermediate zone, there is no detectable presence of exfoliation material. In a peripheral zone, there are numerous large, bush-like deposits, compared to central zone, which can be detached from the lens surface. Posterior synechiae formation is also possible between lens capsule of the iris and posterior iris surface. This might represent an extra exfoliation material. In pre-equatorial zone brush like and amorphous deposits might be present, which are localized between the capsule and the epithelium of the lens. Vertical striations of the bundles of exfoliation fibers might be detectable in this zone. It is suggested that these bundles are originated from pits which are part of epithelial cells of the lens. Exfoliation material covers zonules between the lens and ciliary processes. As shown by electron microscopy

studies, the zonular material is replaced with the exfoliation material near to their attachment to the ciliary epithelium. Zonules might also become infiltrated.

In iris exfoliation material is found on the anterior and posterior surface, as well as in the iris stroma[40]. On the posterior surface, exfoliation material is manifested as a variably thick, continuous layer. This layer corresponds the location of the basement membrane of the posterior iris epithelium. By electron microscopy, the posterior epithelium of the iris is characterized with the degenerative changes. Exfoliation fibers originate from the vacuoles of epithelial cells. Degeneration is also detectable in the dilator and sphincter muscles. With regards to anterior surface of the iris and crypts, it may contain exfoliation material in the stroma in case of advanced XFS. These exfoliation deposits might well be attached to the pupillary border. Immunohistochemical investigation shows deposits of exfoliation material in deeper stroma, including adjacent to fibroblasts and melanocytes. Significant perivascular deposition of exfoliation material in iris blood vessels is also found, which sometimes represent the first microscopic sign of XFS, observed by light microscopy[41]. Histochemical stain for lectin reveals the presence of glycol conjugates of exfoliation matrix in sub-endothelial zone in the blood vessels, which resembles the deposits in the posterior and anterior parts. These exfoliation deposits are also associated with the degenerative changes in the smooth muscle cells, vascular basement membranes and endothelial cells. This finally results in the loss of vascular cellularity.

In ciliary body, brush-like feathery processes and/or amorphous exfoliation material is detected by examination with light microscopy. The mentioned deposits are localized in the basement membrane of the ciliary epithelium. With regards to electron microscopy, exfoliation fibers are revealed in the basement membranes and in the degenerating epithelial cells.

The deposition of exfoliation material is detected in *corneal epithelium light* as well as by electron microscopy. Endothelial involvement results in the thickening of Descemet's membrane and moderate loss of endothelial cells. This in turn can cause the decompensation of cornea in case if intraocular surgery is necessary. Deposition of extracellular material is also visible along Schlemm's canal and in the inter-trabecular spaces. In addition, melanin deposition can be present. Trabecular beams are gradually thickened, which compresses intertrabecular spaces. These process is known to cause the progression of the XFG. More exfoliation material is detected within the juxtacanalicular tissue near to the inner wall of Schlemm's canal by electron microscopy. Similar vacuoles are detected in endothelial cells. Therefore, it has been suggested that these vacuoles are produced by the canal lining endothelial cells. Trabecular cells may fallout. This also plays a role in the development of exfoliation glaucoma. In some patients, corneal endothelial cells also migrate across the Schwalbe's line above the trabecular beams. It is thought that this migration contributes the reduced aqueous outflow[42].

1.3 Biochemistry of exfoliation syndrome

Even though there is an ongoing extensive research efforts, the precise biochemical and chemical composition of exfoliation material is not known. Histochemical and immunohistochemical studies suggest that, exfoliation material represents glycoprotein/proteoglycan complex, which is surrounded by glycoconjugates, glycosaminoglycans, including chondroitin sulfate, heparin sulfate, dermatan sulfate and hyaluronan. To date, as the key biochemical change which is thought to be associated with the deposition of the exfoliation material is considered overproduction and abnormal metabolism of glycosaminocligans and proteoglycans[43]. Moreover, recent biochemical data, examining aqueous liquid from patients with XFS,

demonstrates higher levels of hyaluronan and dermatan/chondroitin sulphate[39]. Protein components of the exfoliation material bear the elastic fiber epitopes of and noncolagenous basement membrane proteins. These proteins include, latent TGF-β binding protein 1/2, fibrillin 1, elastin, amyloid P, emilin, microfibril associated protein 1 and vironectin. These kind of depositions suggest that XFS might represent the type of aging elastosis.

Fibrillin microfibrils represent the major component of elastic fibers. They appear as beads-on-string shape and conferring long range extensibility and elastic deformation of the tissues. These fibrils play a major role in tissue homeostasis. They interact with transforming growth factor-β (TGF-β) and many other factors, including cell surface receptors, such as integrins[42]. From the three fibrillin isoforms, fibrillin 1 represents the most widely distributed isoform in human tissues. Fibrillin 1 represents a glycoprotein, which is composed of 2871 amino acids and it's molecular mass weighs of ¬320kDa[43]. Fibrillin 1 glycoprotein is composed of 59 domains, including N-terminal region, epidermal growth like domains, TGF-β binding like domains and hybrid domains. Supposedly, these domains, are characterized with the linear, rod-like structure[44]. Fibrillins assemble into microfibrils. The exact arrangement of fibrillin monomers into the microfibrills is not yet understood. Fibrillin microfibrils have a diameter of ¬10-12nm[45].

Fibrillin is characterized with the wide distribution in connective tissues and it has been shown to be part of the most tissues and the zonular fibers of the normal human eye. There is an extensive network of fibrillin fibers in cornea, trabecular meshwork, ciliary body, iris, lens, in conjunctiva and in the ciliary body stroma[46]. Therefore, it appears to be the main structural element of the human eye. It has a role in elastogenesis and also involved in extracellular matrix organization, by providing structural system that links elastic fibers and to other matrix components. With regards

to non-elastic tissues, they perform an anchoring function. Particularly higher load of fibrillin fibers are found in areas of high mechanic stress[46].

Schlötzer-Schrehardt and colleagues studied in detail the distribution of fibrillin 1 in the eye from patients with XFS. They found a strong immunoreaction for fibrilin 1, which was localized in pseudoexfoliation material in all intraocular sites and extraocular konjuctival and skin specimens in addition to normal fibrillary network[46]. These finding indicates an increased and generalized production of abnormal fibrillin 1 containing material in patients with XFS. By electron microscopy study, gold marker staining clearly demonstrated its association with exfoliation material and their microfibrillar subunits. The structure of the microfibrillar subunits of exfoliation material closely resembles of the structure of connective tissue microfibrils, which have a diameter of 10 to 12 nm, a tubular cross-section, and an average but variable periodicity of 50 to 55 nm[47]. Interestingly, both immunohistochemical and electron microscopic studies indicated that fibrillin 1 is accumulated solely in extracellular exfoliation material and intracellular structures or cytoplasmic vesicles are intact. This suggests that exfoliation material formation is an extracellular event. In addition, the normal fibrillin containing inclusions in lens capsule is much larger in XFS patients compared to patients without disease.

Another important molecule which has been implicated in the development of XFS is TGF-β1. TGF-β1 represents the member of a large superfamily of growth-modulating polypeptides. These polypeptides modulate extracellular matrix formation[48]. Dysregulation of TGF-β1 expression is shown to be involved in various diseases, which are accompanied with pathological production of the matrix. TGF-β1 is normally secreted as biologically inactive or latent form. The latent form of TGF-β1 contains three major components, including mature TGF-β1 dimer, latency-associated peptide (LAP), and latent TGF-β1 binding protein (LTBP), which forms the large latent TGF-β1 complex. For the biological activation of TGF-β1, it's dissociation from LAP is

necessary. As for LTBP, it is involved in the assembly and secretion of latent TGF-β1. It is also involved in latent TGF-β1 activation and targeting for storage in the extracellular matrix[49]. LTBPs are the family of glycoproteins which are characterised with the structural similarity to the fibrillins. There are four major isoforms of LTBPs described so far. Schlötzer-Schrehardt and colleagues studied the expression of various isoforms of LTBP and TGF-β in anterior segment tissues of XFS patients and control eyes on the protein and mRNA level, including light microscopy immunohistochemistry, in situ hybridisation and semi-quantitative RT-PCR. Measuring TGF-β1 and TGF-β2 levels in aqueous humor and serum of XFS and control patients by ELISA method, showed the significantly increased concentrations of total and active TGF-β1 in aqueous humour in XFS patients with or without glaucoma, compared to control eyes, whilst the level of TGF-β2 was not significantly different. In addition, the mRNA and protein expression of LTBP-1 and 2, and TGF-β1, but not TGF-β2 was significantly increased in the tissues of anterior segment of XFS eyes. This increase was more pronounced in the non-pigmented epithelium of the ciliary body. Immunohistochemical staining for latent TGF-β1 was associated with deposits of exfoliation material[50].

It is known that TGF-β1 induces endothelin-1 synthesis and that endothelin-1 further stimulates ROS production. Endothelin-1 represents 21-amino acid peptide which shows the mitogenic characteristics. Endothelin-1 is considered as one of the potent vasoconstrictors in the human body. Endothelin-1 is involved in the modulation of ocular blood flow in the human eye. It also regulates ocular blood pressure by two mechanisms, mainly by the contraction of trabecular meshwork and to a lesser degree by the contraction of the ciliary muscle via endothelin-1 receptor activation[51]. Endothelin-1 is excessively secreted by non-pigmented ciliary epithelial cells. The endothelin-1 concentration in the aqueous humour is 3 to 4 times higher compared to its plasma levels[52]. In in vivo experimental models laser stimulation of the uveal tissues

induces the production and release of endothelin-1 into the aqueous humour, which in turn causes significant increase in intraocular pressure[53]. Endothelin immunoreactivity is also higher in aqueous humour in patients with primary open angle glaucoma and cataract compared to patients with only cataract and controls[54]. Koliakos and colleagues investigated the level of endothelin-1 in aqueous humour from patients with XFS and exfoliation syndrome glaucoma. The results of their study, showed that mean endothelin-1 concentration in XFS aqueous samples was 4.6 (SD 2.3) pg/ml and it was significantly higher compared to age matched control samples (2.8 (SD 1.71) pg/ml); ($p = 0.006$). In addition, the total protein concentration was significantly elevated in the XFS samples (0.380 (SD 0.159) v 0.279 (SD 0.144) mg/ml in the controls), ($p = 0.023$). The results from the study of suggested an important role of the increased concentration of endothelin-1 in the aqueous humour of XFS patients in the pathobiology of XFS[54].

ROS (Reactive Oxygen Species) represent the chemically reactive oxygen containing species. The examples of ROS are superoxide, peroxides, hydroxyl radical, singlet oxygen, and alpha-oxygen. They are accumulated inside the cells as a result of a various normal metabolic processes, as well as during different diseases and as the result of environmental stress[55]. When the concentration of the ROS exceeds the concentration of antioxidant molecules, including catalase, superoxide dismutase, glutathione peroxidase and small molecules (Vitamin C, E), oxidative stress occurs[56]. Oxidative stress plays a significant role in many diseases, including XFS and XFG. Koliakos and colleagues first described that the concentration of the antioxidant ascorbic acid was reduced in the aqueous humor in patients with XFG[57]. It has been hypothesized that oxidative stress mechanisms in ocular tissues play major role in the pathogenesis of XFS[58]. DNA oxidation generates base and sugar group adducts, causes DNA single and double strand breaks, as well as cross linking with other molecules. Sorkhabi and colleagues assessed the presence of 8-hydroxy-2′-deoxyguanosine

(8OHdG), which is generated upon oxidative stress in aqueous humour and in serum in patients with cataract surgery with or without XFS. They found out that, mean 8OHdG concentration in the PEX aqueous (3.34±1.93 ng/ml) and serum (17.63±6.78 ng/ml) samples were significantly higher than that measured in the control aqueous (1.98±0.70 ng/ml) and serum (13.63±3.54 ng/ml) samples, respectively (P=0.002, P=0.010). Which suggests that oxidative DNA damage plays an important role in the pathogenesis of XFS[59]. It is known that ROS further activate TGF-β biosynthesis and also stimulate matrix metalloproteinase (MMP) activities, such as MMP2 and MMP9[60]. MMPs are enzymes, which are capable of degrading all kinds of extracellular matrix proteins. In addition, they contain a of bioactive molecules. MMPs are also thought to play a major role in cell proliferation, migration, differentiation, angiogenesis, apoptosis and host defence[60]. It has been also shown that MMPs and their inhibitors are significantly increased in aqueous humours in patients with XFS and XFG[61]. ROS can also induce the synthesis of additional growth factors and cytokines, which in turn attenuates tissue damage. It has been shown that ROS modulates the expression and function of vascular epithelial genes and stimulate the sectretion of vascular endothelial growth factor (VEGF), the basic fibroblast growth factor (FGF) and the platelet-derived growth factor (PDGF). They are involved in the regulation of fibroproliferation, in cell growth, differentiation and cell survival[62]. Biochemical data from Koliakos and colleagues indicate the increased growth factor activity in aqueous humour of patients with XFS[63]. Which might be the reflection of the increased ROS production.

Another important factor implicated in XFS and XFG pathogenesis in homocysteine. Homocysteine represents a non-proteinogenic α-amino acid, which is homologue to amino acid cysteine. Homocysteine is biosynthesized from methionine and it can be recycled into methionine or converted into cysteine with the help of B-vitamins. High blood levels of homocysteine is related to the endothelial cell injury,

blood vessel inflammation and therefore atherogenesis, which in turn can cause ischemic injury. In addition hyperhomocysteinemia is associated with early pregnancy loss and with neural tube defects[64,65,66]. In addition, it has been observed that increased levels of plasma homocysteine is associated with XFS and XFG. The results from the study of Vessani et al, showed that homocysteine levels were higher in XFS patients compared with controls (XFS: P= .003; XFG: P = .009); levels in normal-tension glaucoma were higher than in controls but this difference was not statistically significant (P = .2). Hyperhomocysteinemia was shown in 16 of 25 (64%) XFS and 28 of 50 (56%) XFG patients, whilst it was seen in 13 of 25 (52%) normal- tension glaucoma patients, and 7 of 24 (29.2%) controls (P = .005). Multiple logistic regression analyses comparing XFS and XFG patients with controls indicated that elevated plasma homocysteine concentration was a significant risk factor for XFS, in both those patients (odds ratios per 1.0 μmol/l increase in plasma homocysteine concentrations =1.47; 95% confidence interval [CI] = 1.08–2.0) and in XFG patients (odds ratio = 1.3; 95% CI = 1.07–1.6). XFG and normal-tension glaucoma patients did not show significant difference with respect to hyperhomocysteinemia. However, logistic regression modelling of XFG vs normal-tension glaucoma patients showed that an increased homocysteine concentration represented the significant risk factor for XFS in the presence of glaucoma (odds ratio per 1.0 μmol/l increase in homocysteine = 1.2, 95% CI = 1.0–1.4). These relationships were not affected by adjustment for potential confounding due to gender, history of hypertension, or other factors[34].

In summary, current data supports the idea that production of ROS whether caused by UV irradiation or other mechanisms, plays an important role in the development of XFS and XFG. The increased production of ROS can create the vicious cycle of the ROS-cytokine-extracellular matrix molecule accumulation. In addition, increased homocysteine levels have been shown to be related to coffee consumption,

so the dietary habits might also play the role together with the specific genetic background discussed in the following chapters.

1.4 Association of XFS with systemic diseases

Extraocular deposits of exfoliation material have also been detected in visceral organs and in connective tissue, in close association to fibroblasts, striated and smooth muscle cells. In addition, exfoliation material has been also detected in heart muscle cells. The evidence with regard to the fact that such a deposits can cause the degeneration of the extraocular tissues is not direct. However, some studies show that XFS is associated with cerebrovascular and cardiovascular morbidity[67]. The literature is full of controversial findings. For example, according to the investigation of Citiric et al., performed in 50 patients, it has been shown that in patients with XFS the prevalence of coronary artery disease (CAD) is significantly higher[68]. Similar to Citiric and colleagues, French at al., also discovered the significant association of XFS and XFG to CAD, including cardiomyopathy, aortic aneurism and different stages of ischemic heart disease[69]. On the contrary, no association between XFS and XFG to CAD has been found by the studies from Emiroglu et al.,[70] and Tarkkanen et al.[71] Nevertheless, some studies report that ischemia and decreased blood flow is associated with the XFS. The mechanisms of CAD in XFS patients might be the deposition of pseudoexfoliation material in the vascular walls, which in turn increases the vascular resistance and decreases blood flow. In some tissues the local ischemia and atherosclerosis was correlated with elastosis[72].

XFS has been frequently described in patients with transient ischemic attack[73]. The magnetic resonance imaging shows higher incidence of white matter hyperintensities, which in turn represent the ischemic changes, in patients with the diagnosis of XFS[74]. In addition, some studies have shown that the blood flow velocities

is significantly decreased in the middle cerebral artery, in patients with XFS and XFG[75], which in turn causes the decreased brain perfusion in XFS patients. Some studies have also noted that chronic cerebral diseases, including cerebral atrophy, senile dementia and chronic cerebral ischemia is more common in patient with XFG, compared to patients with primary open angle glaucoma. In some, but not all studies XFS was also associated with the development of Alzheimer's disease. Systemic endothelial dysfunction, elevated homocysteine levels, arterial hypertension and aortic aneurisms have also been seen in association with XFS and XFG[76].

In summary, ample of evidence suggests that XFS and XFG might be the risk factors of CAD and cerebrovascular diseases. On the other hand, XFS and systemic vascular diseases might share common pathologic mechanisms and XFS may be a local finding of systemic pathological processes.

1.5 Genetics of Exfoliation Syndrome

The notion about the genetic background of XFS and XFG comes from the fact that the disease is characterised with the significant variability in geographical distribution, as discussed above. Nowadays, the main tool to study the genetic background for XFS is genome wide association studies (GWAS). For this reason, the basic principle of this technology will be first discussed, followed by the description of the current knowledge about the genetic determinants of XFS and XFG.

Genome wide association studies (GWAS). GWAS test is used to test hundreds to thousands to millions of genetic variants across the genomes of many individuals to detect genotype-phenotype associations[77]. Genotyping represents the most common approach for the profiling of genetic data by GWAS. Genotyping arrays are using fluorescence and DNA hybridization technologies for the detection of the single-

nucleotide polymorphisms. During this process, several probes are placed on the array. The hybridization efficiency of one single-nucleotide polymorphism allele is substantially different from the other allele(s) for any given probe[77]. Single nucleotide polymorphism (SNP), is the unit of genetic variation. SNPs represent the single base-pair changes in the DNA sequence. Such a changes occur with high frequency in the human genome[78]. In genetic studies SNPs represent the markers of a genomic region. Most of such SNPs have almost no impact on the organism. However, in some cases SNPs can also cause functional changes, including changes in mRNA transcript stability, amino acid changes, and changes to transcription factor binding affinity[78]. SNPs represent the most frequent form of the genetic variation in the human genome and they are present in large proportion of human populations [79]. Usually, SNPs are represented by alleles. This means that within a population there are two commonly occurring base-pair possibilities for one SNP location. The frequency of a SNP is given in terms of the less common allele frequency or minor allele frequency. For example, the SNP with the frequency of minor allele (G) 0.30 shows that 30% of the population has G allele versus the more common allele (the major allele) which found in 70% of the population[78]. Some SNPs cause the change in protein function which then causes the disease.

Linkage disequilibrium (LD) represents the property of SNPs on a contiguous stretch of genomic sequence that describes the degree to which an allele of one SNP is inherited or correlated with an allele of another SNP within a population[78]. The term was introduced from population geneticists to mathematically describe changes in genetic variation within a population over time[78]. Different human subpopulations have different patterns and degrees of linkage disequilibrium. Linkage disequilibrium is a common property used in GWAS studies. GWAS takes advantage of linkage disequilibrium structure, to genotype only one or a few of the correlated variants in the haplotypes and offers clues about causal disease-associated variants[80].

LOXL1 – Lysil oxydase like protein 1 gene polymorphisms in XFS and XFG.

According to the information from entrez.gene LOXL1 represents the member of the lysil oxidase family of proteins, including other four members (LOX and LOX 2-4). LOXL1 is encoded by LOXL1 gene, which is located at 15q24.1 chromosome. Overall function of the members of LOXL1 family is to participate in the structural integrity of various human tissues. With regard to LOXL1, it is expressed in all ocular tissues except the retina. It represents the prototypic member of the family and is essential for the biogenesis of connective tissue. Particularly, it represents an extracellular copper-dependent amine oxidase, which catalyses the first step in the formation of the collagen and elastin cross links. LOXL1 gene encodes preproprotein, which is proteolitically processed and generates mature enzyme. LOXL1 have 7 exons, a highly conserved C-terminus and poorly conserved N-terminus[81]. It's C-terminal sequence is sufficient for amine oxidase activity, whilst N-terminal domain has an additional function in the regulation of development, senescence, tumor suppression, cell growth control and chemotaxis to each member of the family. LOXL1 is essential factor for the renewal of elastic tissues. Liu et al. observed the strong co-localisation of LOXL1 with elastin in vivo[82]. They also conducted an experiments with LOXL1-knockout mice and showed that these mice develop multiple abnormalities of elastic tissues, including diverticula, lax skin, enlarged airspaces, and prolapse of the pelvis and rectum. Female mice which are lacking LOXL1 gene, from the beginning have the normal appearing elastic tissue. However, after pregnancy and birth they are not able to compose new elastic tissues. Collectively, these data suggest that LOXL1 is responsible for the targeted renewal of elastic fibers[82]. At the molecular level, LOXL1 polymerizes tropoelastin monomers into growing elastin polymers[82]. For this, LOXL1 is first targeted to sites of elastogenesis. This is initiated by its pro-region, which is located near to its N-Terminus[79]. For the activation of LOXL1, the removal of the pro-region by a proteinase is necessary. This cleavage finally activates the catalytic domain of LOXL1, which is located towards the C-terminus of LOXL1. After activation, LOXL1 causes the deamination of the lysine

residues in the tropoelastin molecule, which allows to the formation of elastin polymer[83].

The gene mutations are primarily known by their association with the development of XFS and XFG. First evidence of the association of LOXL1 gene polymorphisms with XFS and XFG comes from the pioneering study from Thorleifsson and colleagues, conducted in 2007, using GWAS technology. According to their study, it has been shown that three SNPs are highly associated with XFG and XFS. All these three SNPs are located within the *LOXL1* gene. One SNP, rs2165241, is located in intron 1 of *LOXL1*. The other two SNPs, rs1048661 and rs3825942, represent the missense variants located in exon 1. For both coding SNPs, the 'G' allele was associated with a higher risk of XFS and XFG[84]. The associations of LOXL1 gene polymorphisms with XFS and XFG are now studied in various different populations, which are outlined in table 2.

Table 2. Summary of the genetic association of two coding variants in LOXL1 gene with XFS/XFG. *Adapted from Whigham et al., Saudi Journal of Ophthalmology, 2011*

Studied population	rs1048661 'G' allele		Significant association	rs3825942 'G' allele		Significant association	References
	Case	Control		Case	Control		
Icelandic	0.781	0.651	Yes	0.984	0.847	Yes	Thorleifsson et al. (2007)
Swedish	0.834	0.682	Yes	0.995	0.879	Yes	Thorleifsson et al. (2007)
American	0.819	0.6	Yes	0.986	0.88	Yes	Fingert et al. (2007)
American	0.787	0.665	Yes	0.939	0.844	Yes	Challa et al. (2008)

American	NA	NA	NA	1	0.856	Yes	Yang et al. (2008)
American	0.843	0.703	Yes	0.959	0.798	Yes	Aragon-Martin et al. (2008)
American	0.829	0.719	No	0.988	0.795	Yes	Fan et al. (2008)
Australian	0.78	0.66	Yes	0.95	0.84	Yes	Hewitt et al. (2008)
Austrian	0.841	0.671	Yes	0.994	0.817	Yes	Mossbock et al. (2008)
German	0.844	0.66	Yes	0.992	0.856	Yes	Wolf et al. (2010)
German	0.818	0.644	Yes	0.951	0.857	Yes	Pasutto et al. (2008)
Finnish	0.825	0.683	Yes	0.968	0.823	Yes	Lemmela et al. (2009)
Italian	0.825	0.693	Yes	1	0.821	Yes	Pasutto et al. (2008)
Saudi Arabian	0.876	0.762	Yes	0.968	0.817	Yes	Abu-Amero et al. (2010)
Indian	0.721	0.634	No	0.923	0.742	Yes	Ramprasad et al. (2008)
Chinese	0.542	0.444	No	0.992	0.918	Yes	Lee et al. (2009)
Chinese	0.11	0.48	Yes	1	0.9	Yes	Chen et al. (2009)
Japanese	0.036	0.493	Yes	1	0.877	Yes	Fuse et al. (2008)
Japanese	0.008	0.46	Yes	1	0.857	Yes	Hayashi et al. (2008)
Japanese	0.006	0.45	Yes	0.994	0.853	Yes	Mabuchi et al. (2008)
Japanese	0.005	0.474	Yes	0.995	0.85	Yes	Mori et al. (2008)
Japanese	0.005	0.497	Yes	0.986	0.863	Yes	Ozaki et al. (2008)
Japanese	0.005	0.554	Yes	0.993	0.806	Yes	Tanito et al. (2008)
South African	0.99	0.81	Yes	0.13	0.62	Yes	Williams et al. (2010)
South African	1	0.883	Yes	0.14	0.617	Yes	Rautenbach et al. (2011)

Overall it is accepted that these two common non-synonymous protein-coding variants in exon 1, rs1048661G>T (Arg141Leu) and rs3825942G>A (Gly153Asp), and one intronic variant (rs2165241T>C), conferring a 20-fold increased risk for XFS[85]. The association between the 'G' risk alleles and XFS has been largely consistent across all tested populations. However, there have been important exceptions. For example, the 'G' allele for rs1048661 was not significantly associated with XFG in an Indian[86] and Chinese[87] population. Furthermore, the 'G' allele of rs1048661 was found to be protective in another Chinese population[88] and several Japanese populations[89]. The inconsistent association between XFG and rs1048661 was also observed for the intronic SNP, rs2165241. For this reason, it was concluded that this SNP is not a functional variant for XFS/XFG[90]. Interestingly, recent large scale study from Aung and colleagues, which included different populations across six continents, including Georgia, found that no common variant in LOXL1 was consistently associated with XFS across all the collections, and no common variant in this gene surpassed genome-wide significance in random effects analysis[17]. In addition, they also identified the rare nonsynonymous variant rs201011613[A>T], was associated with the decreased risk of XFS[17].

How the SNPs in LOXL1 gene cause the XFS and XFG still remains the subject of investigation. Several SNPs have been investigated for their effects on LOXL1 expression. Both coding SNPs identified in the initial GWAS were studied for effects on LOXL1 expression. The coding SNP rs1048661 was associated with altered expression of LOXL1. Its 'G' allele was associated with a 7.7% decrease of LOXL1 expression in adipose tissue[91] and approximately 20% in post-mortem ocular tissue[91]. In contrast, the most strongly associated SNP, rs3825942, was not associated with an increase or decrease of LOXL1 expression in adipose or ocular tissue[92]. Another SNP, rs16958477, was found to alter LOXL1 expression in vitro[92]. This SNP is located 659 base pairs upstream of LOXL1. It appears to influence the promoter activity of the one

kilobase region upstream of LOXL1. Its 'C' allele was associated with increased promoter activity in a commercial plasmid. This SNP was originally investigated for a connection to pelvic organ prolapse but no significant correlation was observed[92]. The SNP also did not associate with XFG in black South Africans[18]. The 'A' allele did associate with XFS in a large Caucasian cohort with an OR 2.05 (1.54–2.72). However, this association was much weaker than rs3825942, which yielded an OR of 25 (8.3–50) in the same cohort[90]. Recent study from Pasutto et al., also demonstrated that another SNP found in XFS patients, such as rs11638944:C>G transversion exerts a cis-acting effect on the expression levels of LOXL1, mediated by differential binding of the transcription factor RXRα (retinoid X receptor alpha) and by modulating alternative splicing of LOXL1, eventually leading to reduced levels of LOXL1 mRNA in cells and tissues of risk allele carriers[85]. These finding may uncover a functional mechanism by which common noncoding variants influence LOXL1 expression. Another possible mechanism is that the variants can have an effect on LOXL1 splicing. However, this subject requires further studies.

Interestingly, some studies show that LOXL1 may be a target of autophagy system. Autophagy plays a central role in the development of many age related diseases, and it appears to have a role in the development of XFS as well[93]. A common finding in many age-related diseases is the dysfunctional cellular degradation of misfolded proteins by the proteasome and / or toxic aggregates and organelles by the autophagy system[94]. In addition, the mitophagy component of the autophagy leads to increased oxidative stress and accumulation of depolarised mitochondria in case autophagy fails[94]. The study from Bunney at al., showed that LOXL1 N-terminus contains multiple high disorder probability domains[93]. These domains contribute to the development of protein misfolding and subsequent autophagy. Maximal disorder occurs in a wide span between residues 150 and 170. This span includes position 153, in which G homozygosity is found in 98% of XFG patients[95]. Replacement of glycine by the

negatively charged aspartic acid (D) at this position resultes in a substantial (8-10%) decrease in disorder probability over the 151-180 aa domain. The C-terminus included a 'deep well' of low disorder probability that was highly conserved throughout all the LOX protein family. Finally, the comparative analysis of LOX proteins revealed that while the smaller LOX protein contains domains with disorder probability, none of the other LOX-like proteins displayed any disordered region.

Other genes in XFS and XFG. A worldwide partnership study of the genetic basis for XFS established recently, and 2 GWAS studies involving about 13,838 cases and 110,275 controls from 6 continents discovered significant associations between increased risk of XFS and 6 new gene loci[17]. These genes include FLT1-POMP, CACNA1A, AGPAT1, TMEM136-ARHGEF12, SEMA6A and RBMS3.

CACNA1A gene encodes the alpha 1A subunit of the type P/Q voltage-dependant calcium channel. Calcium channels transport calcium ions across cell membranes. Therefore, they play an important role in a cell's ability to generate and transmit electrical signals. The presence of high concentrations of calcium directly correlates with the presence of XFS fibril aggregates, as shown by electron microscopic studies[96]. It is also known that fibrillin uses calcium in order to form stable aggregates[97]. Therefore, it could be suggested that changing the function of a calcium channel and consequent changes in calcium concentrations, may result in the formation of the aggregates in XFS eyes[97]. Lingala and colleagues conducted the GWAS study on 1,484 XFS patients and 1,188 controls from Japan. In addition, they expanded their study on a further 6,901 patients and 20,727 controls from 17 countries from 6 continents. The study identified the significant association between the new locus (CACNA1A rs4926244) and an increased risk to XFS (Odds ratio [OR] = 1.16, P = 3.36 × 10–11)[98]. Later the same group identified five new loci associated with XFS in the same population of patients[99]. These include POPM, SEMA6A, TMEM136, RBMS3 and

AGPAT1 genes. The study involved 32 countries across six continents, including Georgia.

FLT1 protein is encoded by FLT1 gene. FLT1 belongs to the family of vascular endothelial growth factor receptor (VEGFR). VEGFR proteins represent receptor tyrosine kinases (RTKs). They contain an extracellular ligand-binding region. This region is composed of seven immunoglobulin (Ig)-like domains. In addition, they contain a transmembrane segment, cytoplasmic domain. Their cytoplasmic domain is characterised with the tyrosine kinase (TK) activity. FLT1 binds to placental growth factor, VEGFR-A, VEGFR-B, playing the major role in vasculogenesis and angiogenesis. Expression of FLT1 receptor is detected in placental trophoblast cells, vascular endothelial cells and peripheral blood monocytes. FLT1 gene contains multiple transcript variants, which encode different isoforms. These include shortened, soluble isoforms and full-length transmembrane receptor isoforms. The soluble isoforms are found to be associated with the development of pre-eclampsia. The function of this protein in human eye and in pathogenesis of XFS and XFG is not studied yet.

POMP - the molecular chaperone, which binds to 20S preproteasome components, has the crucial role for the formation of 20S proteasome. The 20S proteasome represents the proteolytically active part of the 26S proteasome complex. The degradation of encoded protein happens before the 20S proteasome complex matures. 5' UTR variant of POMP gene is associated with KLICK syndrome, which represents a rare skin pathology. In the study from Aung and colleagues, immunofluorescent analysis of POMP expression showed its expression in the majority of eye cell types. However, POMP protein level was significantly reduced in ciliary body (33% decrease) and iris (45% decrease) in eye specimens from XFS patients, compared to controls. This study was performed by using immunofluorescence

microscopy and immunoblot analysis[17]. Therefore, we can suggest that this gene is related to the development of XFS.

TMEM136 represents the transmembrane protein. The function of TMEM136 is not known. According to the study immunofluorescence study of Aung and colleagues TMEM136 is localised to the blood vessels and vascular endothelial in the eye. TMEM136 protein level was analysed by immunoblotting in eye tissues from XFS patients. The study results showed that, TMEM136 is significantly decreased in ciliary body (32% decrease) and in iris (26% decrease) compared to its expression in the eyes from the patients without XFX. Similar findings has been obtained by immunofluorescence microscopy[17].

AGPAT1, represents the gene, which encodes lysophosphatidic acid (LPA) converting enzyme into phosphatidic acid (PA). LPA and PA represent phospholipids, which are involved in lipid biosynthesis and signal transduction. The enzyme is found in endoplasmic reticulum. With regard to gene, it is localised in the class III region of the human MHC complex. Mentioned gene has two alternatively spliced variants, which encode the same protein.

An RNA-binding protein RBMS3 is encoded by RBMS3 gene. This protein belongs to the c-myc gene single-strand binding protein family, which are characterized by the presence of two sets of ribonucleoprotein consensus sequences (RNP-CS). These sequences contain conserved motifs, RNP1 and RNP2, which were primarily described in RNA binding proteins. Mentioned proteins are involved in gene transcription, DNA replication, apoptosis and cell cycle progression. Protein is mostly localised in cytoplasm. Therefore, we can suggest that it is involved in a cytoplasmic controlling of RNA metabolism and not in transcription. RBMS3 gene is characterised with multiple alternatively spliced variants. These spliced variants encode various isoforms of the protein.

The transmembrane semaphorin - SEMA6A - is required for the development of the thalamo-cortical projection. Therefore, it is widely expressed in developing neural tissue. The above mentioned genes are still the subject of further studies in the pathogenesis of XFS and XFG.

Chapter 2. Materials and Methods

2.1 Study Population and Design

Eight hundred sixty-three self-reported Georgian subjects referred for cataract surgery were recruited between 2015 and 2019 at an ophthalmology tertiary care center "Chichua Medical Center Mzera" LLC. After signing informed consent patients underwent detailed ophthalmic examination, including autorefraction, keratometry, visual acuity check, tonometry, slit lamp examination, A-scan or biometry for IOL calculations, Humphrey 24-2 perimetry, optic nerve head (ONH) and retinal evaluation. Subjects with XFS exhibited deposits of exfoliation material on pupillary margin or lens capsule upon slit lamp examination. Subjects with glaucoma had characteristic changes of ONH, including increased vertical cup-to-disc ratio, retinal nerve fiber layer thinning, neuroretinal rim notching or hemorrhages. Patients over age 60 with evident XFS were included into the case group which was further subdivided into XFS only and XFG subgroups. The control group comprised patients over age 60 with no evidence of exfoliations and glaucoma upon clinical examination. Patients having uveitis and either primary open angle or neovascular glaucoma were excluded from the study.

All patients underwent standard blood tests and health evaluation by the general practitioner (GP) before surgery including Complete Blood Counts (CBC), blood glucose levels, infectious diseases, including hepatitis B and C, HIV and RPR. Electrocardiography (ECG) was also performed if needed as determined by the GP. Non-ocular comorbidities like diabetes, hypertension, ischemic heart disease, myocardial infarction (MI) and stroke were noted.

2.2 Cataract Surgery

All patients were scheduled for standard phacoemulsification with IOL implantation. The surgeries were performed by two surgeons of our center (only in cases where genetic study was done). The standard procedure involved the formation of two side-ports and a 2.75 mm main incision, a continuous curvilinear capsulorhexis, phacoemulsification with Infiniti phacomachine (Alcon, USA) with Ozil handpiece, bimanual removal of cortical material, IOL implantation and wound closure with hydration. In cases of very small pupils, iris retractors were used to improve visualization. In cases of capsular instability capsular tension ring (CTR) was implanted to support the lens, capsular hooks were also used when extreme subluxation was observed. In cases of extensive zonular insufficiency, the eye was left aphakic and subsequent IOL implantation with scleral fixation was scheduled. In cases of vitreous herniation, thorough anterior vitrectomy was performed before wound closure.

2.3 Genetic Study

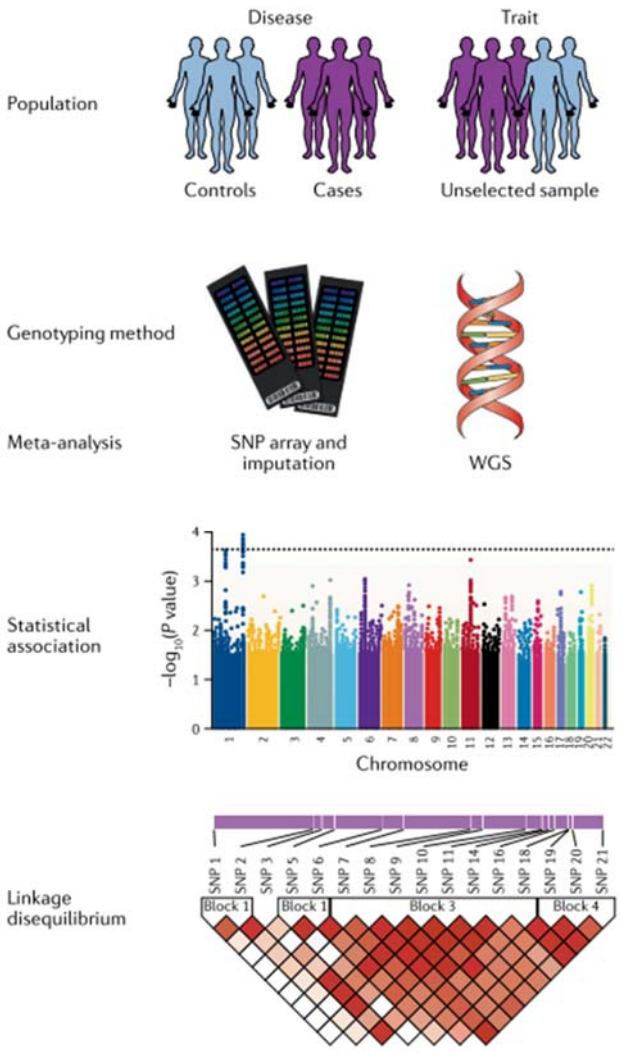
All subjects underwent blood sampling preoperatively. We collected 5 ml of peripheral blood and refrigerated in EDTA-coated tubes before use. All samples were sent for genetic studies to the genetic laboratory of Singapore Eye Research Institute. DNA extraction was performed using a DNA extraction kit according to manufacturer's protocol.

Principal-component (PC) analysis was performed to assess the degree of genetic stratification and population substructure for all samples. PC scores were calculated form a set of unlinked markers. Genetic outliers were excluded from the study. The PC plots were generated using the R statistical software package.

Genome-Wide Association Study (GWAS) was performed using Illumina

OmniExpress Microarray. The process involved DNA typing chips containing a microscopic array of tiny beads covered with single-stranded DNA fragments, synthesized to correspond to different SNPs of human genome. The beads were incubated with previously denatured and fragmented DNA from test subjects. After binding of complementary DNA strands, the beads were incubated with labeled nucleotide mixtures and DNA polymerase to elongate synthetic DNA strands. The labeled nucleotides fluoresced with different colors on the beads making them glow differently. The chips were scanned with high-resolution laser making it possible to identify which alleles were present and whether the person was heterozygous or homozygous for the particular SNP. More than 680 000 SNPs were analyzed for association to XFS.

Figure 1. GWAS Workflow. GWAS is used to detect associations between trait status and genotype or allele frequency. The first step during GWAS study is the identification of the trait or the disease of interest and then selecting the relevant study population (for example, cases and controls for a disease, or an unselected population sample for a trait). Genotyping is performed by using single-nucleotide polymorphism (SNP) arrays, which is combined with imputation or whole-genome sequencing (WGS). Regions of the genome, which are associated with the phenotype of interest at genome-wide significance is identified by association tests. The meta-analysis is a common step to increase the statistical power to detect associations. Causal variants are usually not directly genotyped but are in linkage disequilibrium with the genotyped SNPs. *Adapted from Tam et al., 2019, Nature Reviews Genetics.*



2.4 Ethical Approval

The ethical approval of this study was obtained from David Tvildiani Medical University Ethics Committee. All study procedures were adherent to principles stated in Declaration of Helsinki on Biomedical Research Involving Human Subjects.

2.5 Statistical analysis

Association between SNPs and XFS was tested using logistic regression analysis. SNPs surpassing $P \leq 5 \times 10\text{-}8$ in the GWAS discovery stage were considered associated with XFS. Statistical analysis of our case–control association study was performed using a $\chi 2$ test (Pearson correction). Relative risk association was estimated by calculating odds ratios (OR) along with 95 % confidence intervals (CIs). $p < 0.05$ was considered statistically significant. R statistical software version 2.9.0 was used for analysis.

Chapter 3. Results

3.1 XFS Burden in Cataract Surgery

Eight hundred and sixty-three patients with mean age of 74(±3.7) years undergoing cataract surgery by the author during year 2019 were included into the epidemiological study. Three hundred and seventy–one (57%) were female and 492 (43%) were male. Two hundred and seventy patients were diagnosed with XFS, 117 (46.8%) of them were male and 153 (53.2%) were female. Forty individuals had XFG, 22 (55%) were male and 18 (45%) were female. Five hundred and fifty-three patients had no evidence of XFS. Two hundred and ninety-eight (54%) of them were female and 255 (46%) were male **(Table 3).**

Table 3. Demographic characteristics of patients undergoing cataract surgery at Chichua Medical Center Mzera performed by the author.

Groups		**Male**	**Female**
Total	863	492 (57 %)	371 (43 %)
XFS	310 (36%)	139 (44.8 %)	171 (55.2 %)
XFS only	270 (87%)	117 (43 %)	153 (57 %)
XFG	40 (13%)	22 (55 %)	18 (45 %)
Controls	553 (64%)	255 (46 %)	298 (54 %)

Table 4 and Figure 2 show the total number of patients in general, having technical issues during phacoemulsification. The most common difficulties were inadequate mydriasis, encountered in 17% of all patients, zonular laxity/dialysis in almost 15%% of cases and either hyper-deep or shallow anterior chamber, complicating cataract surgery in 8 to 9% of cases, respectively. Additional surgical manipulations, like CTR and capsular hook use, were necessary in around 15% of all patients. Anterior vitrectomy was performed in 10 patients.

Table 4. Total number of patients having technical difficulties during phacoemulsification.

Difficulty	Number of Patients	% Patients
Small Pupil	151	17
Zonular Laxity/Dialysis	129	14.9
Hyper-deep Anterior Chamber	62	7.2
Shallow Anterior Chamber	80	9.3
CTR	121	14.9
CTR with Capsular Hooks	6	0.7
Anterior Vitrectomy	10	1.2

Figure 2. Percentage of all patients having technical difficulties during phacoemulsification.

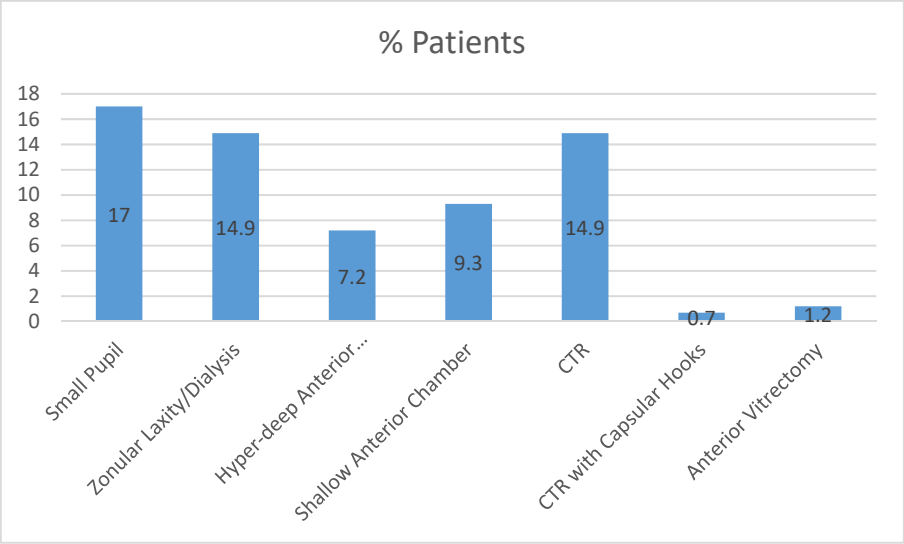
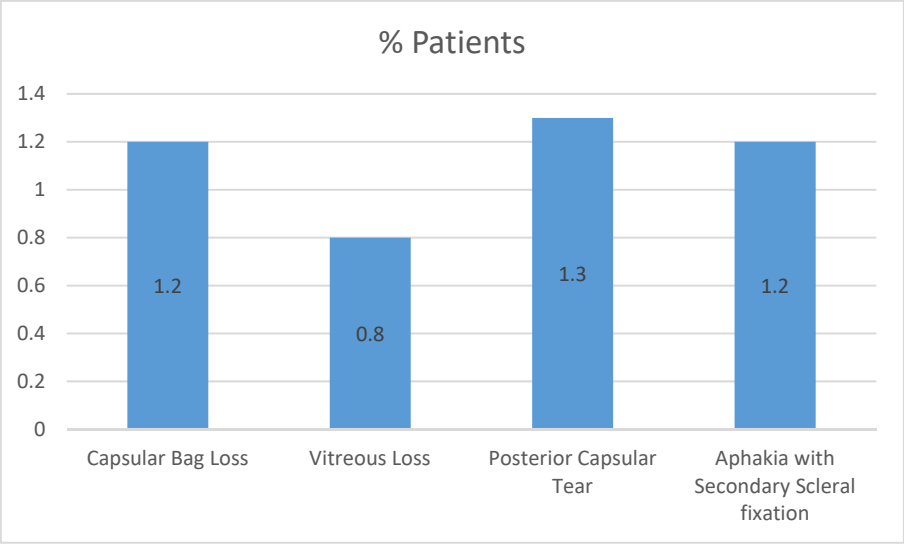


Table 5 and Figure 3 illustrate overall intraoperative complication rate in phacoemulsification. Fortunately, only 1.2% of all patients had unfavorable outcomes needing additional surgery. Capsular bag and vitreous loss, as well as posterior capsular rupture were encountered in around 1% of all patients. Follow-up and evaluation of clinical outcomes was beyond the scope of this work. We only evaluated the technical part of phacoemulsification.

Table 5. Total number of patients having intraoperative complications during phacoemulsification.

Complications	**Number of Patients**	**% Patients**
Capsular Bag Loss	10	1.2
Vitreous Loss	7	0.8
Posterior Capsular Tear	11	1.3
Aphakia with Secondary Scleral fixation	10	1.2

Figure 3. Percentage of all patients having intraoperative complications during phacoemulsification.



The following intraoperative technical difficulties were observed in patients with XFS in contrast to normal controls. Small pupil was seen in 138 (51%) XFS vs 13 (2.3%) normal patients ($p<0.05$, OR 43.4, 95% CI 23.842 to 79.099). Zonular laxity/dialysis of varying degrees was encountered in 121 patients (39%) in contrast to 8 (1.4%) normal individuals ($p<0.05$, OR 56.1, 95% CI 26.836 to 117.4). This was mostly attributable to previous ocular trauma. Hyper-deep anterior chamber made surgery technically difficult in 41 (13%) XFS patients as opposed to 21 (3.7%) patients without exfoliation material seen in the anterior chamber structures ($p<0.05$, OR 1.29, 95% CI 2.671 to 7.99553). In the letter patients, high myopia was the reason of excessive chamber depth. On the other hand, 49 XFS (16%) patients had shallow anterior chambers in contrast to 31 (5.5%) of normal individuals ($p<0.05$, OR 3.8, 95% CI 2.363 to 6.127).

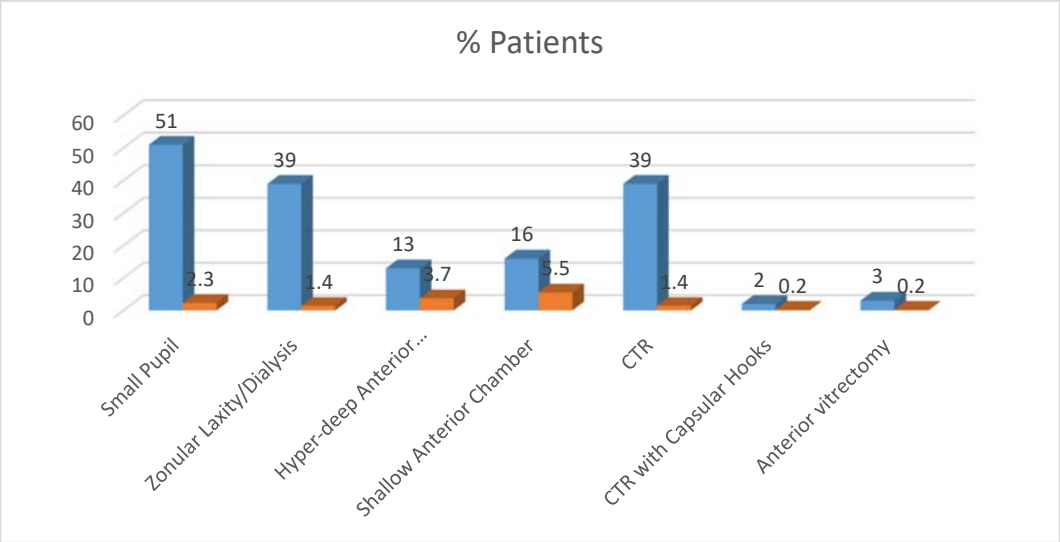
Depending on the extent of zonular dialysis, either capsular tension rings (CTRs) alone (121 patients, 39%) or CTRs together with capsular hooks (5 patients –

2%) were used to stabilize the XFS lenses during surgery. In normal individuals CTRs were implanted in 8 cases, additional capsular hooks were needed in 1 patient ($p<0.05$, OR 56.1, 95% CI 26.836 to 117.422) and ($p<0.05$, OR 10.6, 95% CI 1.233 to 91.214), respectively. Anterior vitrectomy was performed in 9 vs 1 case (OR 19.3795% CI 2.442 to 153.762). The data are presented in Table 6 and Figure 4.

Table 6. Technical difficulties observed during phacoemulsification in XFS vs normal patients.

Difficulty/Complication	Cases N=270 (%)	Controls N=553 (%)	P value	OR (95% CI)
Small Pupil	138 (51)	13 (2.3)	<0.05	43.4 (23.842 to 79.099)
Zonular Laxity/Dialysis	121 (39)	8 (1.4)	<0.05	56.1 (26.836 to 117.4)
Hyper-deep Anterior Chamber	41 (13)	21 (3.7)	<0.05	1.29 (2.671 to 7.99553)
Shallow Anterior Chamber	49 (16)	31 (5.5)	<0.05	3.8 (2.363 to 6.127)
CTR	121 (39)	8 (1.4)	<0.005	56.1 (26.836 to 117.422)
CTR with Capsular Hooks	5 (2)	1 (0.2)	<0.05	10.6 (1.233 to 91.214)
Anterior vitrectomy	9 (3)	1(0.2)	<0.05	19.37 (2.442 to 153.762)

Figure 4. Technical difficulties observed during phacoemulsification in XFS vs normal patients.



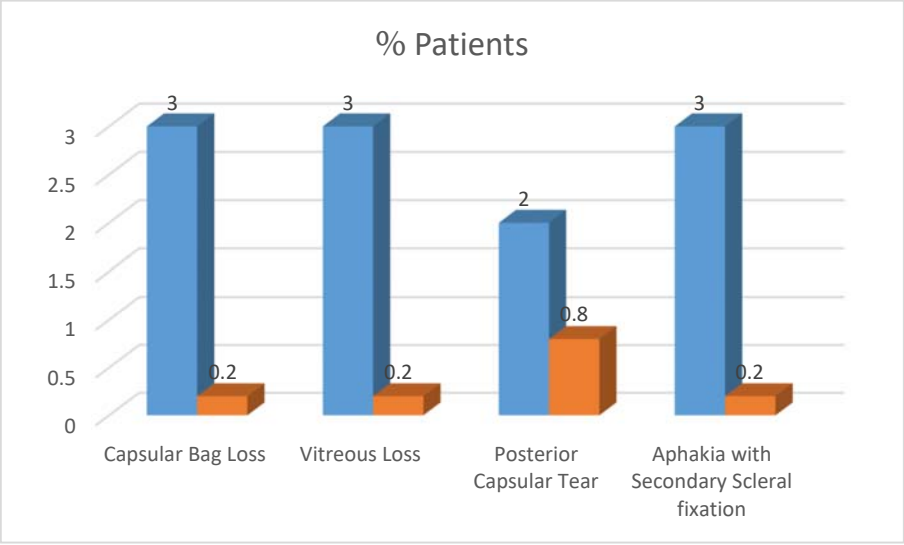
Capsular bag was lost in 9 (3%) XFS patients, vitreous loss was also observed in 6 (2%) XFS cases. This is in contrast to one case of traumatic cataract without XFS where both capsular and vitreous loss was observed ($p<0.05$, OR 19.37, 2.442 to 153.762) and ($p<0.05$, OR 13.8, 95% CI 1.53 to 106.63), respectively. The letter patient was left aphakic and underwent subsequent IOL implantation with scleral fixation. It should be said that all 9 XFS cases had brown subluxated cataracts which is the hardest technical situation in cataract surgery.

Nine XFS patients (3%) vs 1 (0.2%) normal individual ended up aphakic, necessitating secondary IOL implantation with scleral fixation ($p<0.05$, OR 10.6, 95% CI 2.442 to 153.762). Posterior capsular tears were observed comparably in both cases and controls in 6 and 5 patients, respectively. There was no statistically significant difference in both groups. IOLs in all of them were placed into the sulcus, therefore none of those patients needed any additional surgeries. **(Figure 5 and Table 7)**.

Table 7. Intraoperative complications observed during phacoemulsification in XFS vs normal patients

Complication	Cases	Controls	P value	OR (95% CI)
	N=270 (%)	N=553 (%)		
Capsular Bag Loss	9 (3)	1 (0.2)	<0.005	19.37 (2.442 to 153.762)
Vitreous Loss	6 (3)	1(0.2)	<0.005	13.8 (1.53 to 106.63)
Posterior Capsular Tear	6 (2)	5 (0.8)	**>0.05**	2.5 (0.767 to 8.386)
Aphakia with Secondary Scleral fixation	9 (3)	1 (0.2)	<0.05	19.37 (2.442 to 153.762)

Figure 5. Intraoperative complications observed during phacoemulsification in XFS vs normal patients



3.2 XFS and Systemic Vascular Diseases

Two hundred and thirty-six patients with XFS were studied for systemic vascular associations of XFS **(Table 8)**. One hundred and eight (46%) of them were male and 128 (54%) were female. Mean age was 76 (±6.1) years. Two hundred and five patients (87%) had XFS only and 31 (13%) patients were diagnosed with XFG. The control group comprised 250 patients without evidence of exfoliation material upon clinical examination. Seventy-six (30%) patients were male and 174 (70%) were female. Mean age was 70 (±5.1).

Table 8. Demographic characteristics of XFS patients and controls studied for systemic vascular associations

Groups	Total	Male	Female
XFS	236	108 (46 %)	128 (54 %)
XFS only	205 (87%)	91 (44 %)	114 (56 %)
XFG	31 (15 %)	17 (55 %)	14 (45 %)
Controls	150	76 (30 %)	174 (70 %)

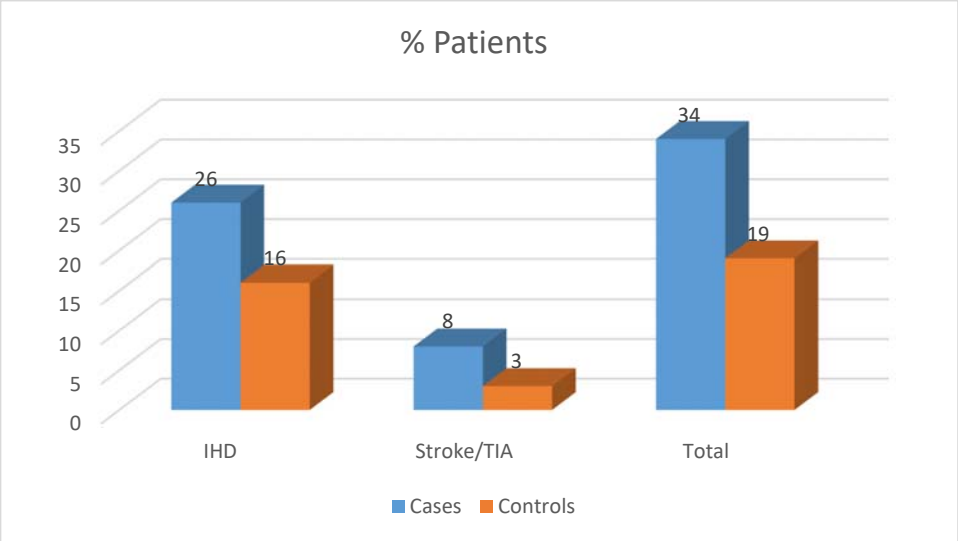
We did not observe any significant association of XFS with arterial hypertension, though we did see clear correlation with its vascular complications. XFS patients were more prone to develop Ischemic Heart Disease (IHD). Sixty-two XFS patients were diagnosed with IHD vs 41 individuals in control group. The presence of XFS conferred about 80% increased risk of IHD (p=0.02, OR 1.8; 95% CI: 1.141 to 2.763). The risk of MI was also significantly elevated in patients with XFS with p=0.05, OR 1.8; 95% CI: 0.99 to 3.604. Significant association of XFS was also observed with Cerebrovascular Disease (CVD) including the history of both strokes and transient

ischemic attacks (p=0.01, OR=3; 95% CI: 1.254 to 7.37). When looking at vascular events in general, we found doubled risk in our XFS patients (p=0.0002 OR=3; 95% CI: 1.454 to 3.327) **(Table 9 and Figure 6)**.

Table 9. Vascular comorbidities in cases and controls

	Cases N=236	**Controls N=250**	**P value**	**OR (95% CI)**
IHD	62 (26%)	41 (16%)	0.02	1.8 (1.141 to 2.763)
IHD with MI	27 (11%)	16 (6%)	0.05	1.9 (0.99 to 3.604)
Stroke/TIA	19 (8%)	7 (3%)	0.01	3 (1.254 to 7.37)
Total	81 (34%)	48 (19%)	0.0002	2.2 (1.454 to 3.327)

Figure 6. Vascular comorbidities in cases vs controls



3.3 Genetics of Exfoliation Syndrome

One hundred and thirty-two patients with XFS were included in the genetic study (**Table 10).** Seventy-three patients (56%) were female and 59 patients (44%) were male. Mean age was 73.7 (± 6.4) years. One hundred and fourteen patients (86%) were diagnosed with XFS only, 18 patients (14%) had XFG. The control group comprised 199 patients without any clinical evidence of XFS or XFG. One hundred and thirty control patients (65%) were female and 69 patients (35%) were male. Mean age was 70.8 (± 7.3) years.

Table 10. Demographic characteristics of XFS patients and controls involved in the genetic study

	Total	**Male**	**Female**
XFS	132	59 (44 %)	73 (56 %)
XFS only	114 (86%)	49 (43 %)	65 (57 %)
XFG	18 (14 %)	10 (55 %)	8 (45 %)
Controls	199	69 (35 %)	130 (65 %)

We identified tree LOXL1 variants in Georgians: rs2165241, rs4886776 (R141L) and rs8042039 (G153D). The former is an intronic, whereas the letter three are exonic variants. The letter two correlate with widely reported rs1048661 and rs3825942, respectively, showing 99% identity. The results are shown in **Table 11**.

The frequencies of widely reported high-risk allele A of rs2165241 were significantly different when comparing cases and controls and associated with XFS (p=0.0001) and they did increase disease susceptibility to approximately 4-fold

(OR=3.8; 95% CI 2.6339 to 5.5802). It was present in 83% of affected individuals, almost 70% of them were homozygous, carrying 6-fold increased risk of disease development (p=0.0001; OR= 5.7; 95% CI: 1.9518 to 16.5321). Heterozygotes had 4.5-fold increased risk (p=0.0001; OR= 4.5; 95% CI: 2.8199 to 7.2454) compared to normal individuals. Interestingly, high-risk allele of rs2165241 was observed in almost 60% of healthy individuals. About a third of them were homozygous.

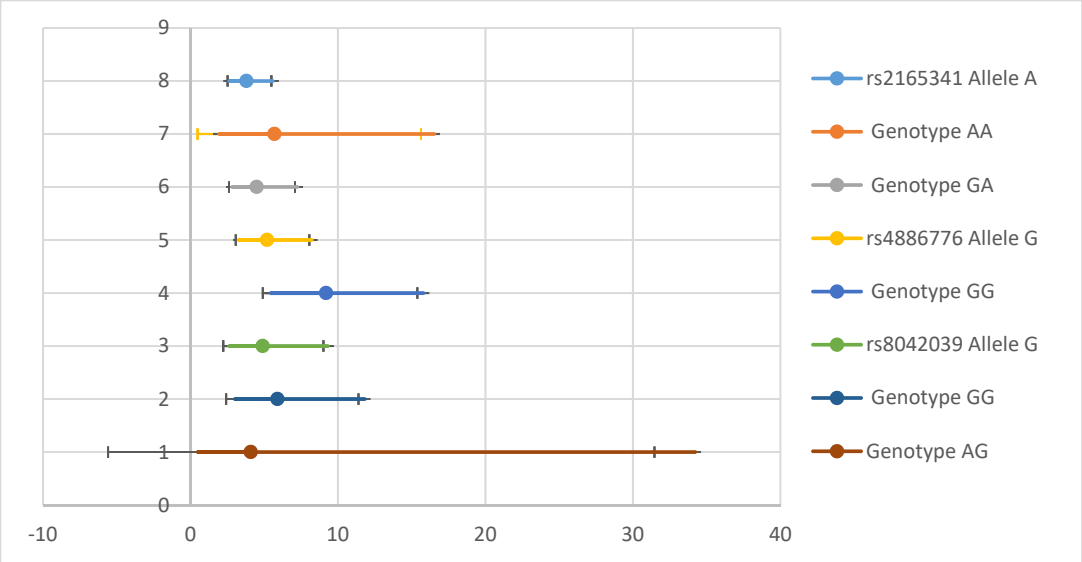
For rs4886776 SNP observed even higher risks associated with G allele, which was present in 90% of affected individuals. It conferred 5-times increased risk of XFS compared to normal controls (p=0.0001, OR=5.2; 95% CI 3.2732 to 8.2217). Interestingly, we did not find statistically significant differences with heterozygotes, whereas in homozygotes, the risk was increased up to 10-fold (p=0.0001; OR=9.2; 95% CI 5.4476 to 15.7981). The G allele was present in two thirds of healthy individuals and half of them were homozygotes.

The same is true for the G allele of rs8042039. It conferred up to 5 times increased risk of XFS as compared to healthy subjects (p=0.0001; OR=4.9; 95% CI 2.6378 to 9.3135). GA carriers did not show any increased susceptibility, whereas GG carriers did, with p=0.0001; OR=5.9; 95% CI 3.0168 to 11.8102. The G allele was present in 80% of normal controls, two thirds of them carried GG genotype **(Figure 7 and Table 11)**

Table 11. Allele and Genotype Frequencies of three LOXL1 variants in XFS and healthy subjects

SNP		Controls % (n=199)	XFS % (n=132)	*P* value	OR (95% CI)
rs2165341					
Allele	A	58.5	82.6	0.0001	3.8 (2.6339 to 5.5802)
	G	41.5	17.4	0.0001	0.2 (0.1387-0.3576)
Genotype	AA	32.2	68.2	0.0001	5.7 (1.9518 to 16.5321)
	GA	52.7	28.8	0.0001	4.5 (2.8199 to 7.2454)
	GG	15.1	3	0.0001	0.2 (0.0313 to 0.2781)
	Total	64/105/30 (AA/GA/GG)	90/38/4 (AA/GA/GG)		
rs4886776					
Allele	G	64.8	90.5	0.0001	5.2 (3.2732 to 8.2217)
	A	35.2	9.5	0.0001	0.2 (0.1216 to 0.3055)
Genotype	GG	32.7	81.8	0.0001	9.2 (5.4476 to 15.7981)
	GA	64.3	17.4	0.19	4.1 (0.4846 to 34.2232)
	AA	3	0.8	0.0001	0.1 (0.0165-1.1782)
	Total	65/128/6 (GG/GA/AA)	108/28/1 (GG/GA/AA)		
rs8042039					
Allele	G	80.9	95.4	0.0001	4.9 (2.6378 to 9.3135)
	A	19.1	4.5	0.0001	0.2 (0.0286 to 2.0209)
Genotype	GG	64.8	91.7	0.0001	5.9 (3.0168 to 11.8102)
	AG	32.2	7.5	0.16	4.1 (0.4846 to 34.2232)
	AA	3	0.8	0.0001	0.2 (0.0233 to 1.6544)
	Total	129/64/6 (GG/GA/AA)	121/10/1 (GG/GA/AA)		

Figure 7. LOXL1 in XFS vs healthy subjects.



We also identified six genes previously reported in association to XFS: CACNA1A rs4926244, POMP rs7329408, TMEM136 rs11827818, AGPAT1 rs3130283, RBMS3 rs12490863 and SEMA6A rs10072088. The results are shown in Table 3.

Among six genes studied, SEMA6A, POMP and CACNA1A were found to be associated with XFS in our population. Previously reported allele G of SEMA6A did not confer any risk in our patients, by contrary, allele A was associated with the syndrome. A allele frequencies of SEMA6A were significantly different when comparing cases and controls and associated with XFS (p=0.001). It did increase disease susceptibility by 80%. It was present in 81% of affected individuals, 65% of them were homozygous. Heterozygotes had almost 2-fold increased risk (*p*=0.001; OR= 1.8; 95% CI: 1.2676 to 2.6973), whereas, homozygotes had the risk of disease development up to 4 times higher (*p*=0.001; OR= 4.0; 95% CI: 1.1531 to 13.9903) than normal individuals. Interestingly, allele A was found in up to 70% of normal individuals and half of them were homozygous.

High-risk allele A of POMP was found only in 16% of XFS cases. The likelihood of disease development rose up to 60% in affected individuals (*p*=0.005; OR= 1.6; 95%

CI: 0.9931 to 2.5634). Heterozygotes did not show any increased risk of disease development (p=0.7; OR= 1.5; 95% CI: 0.0937 to 24.3786) and homozygotes had up to 70% (p=0.06; OR= 1.7; 95% CI: 1.0217 to 2.8713) higher risk when compared to individuals who were not carrying a high-risk allele. Normal individuals carried allele A in 10% of cases and only 0.5% of them were homozygous.

We identified allele G of CACNA1A as high-risk. It was present in about 20% of both affected and normal individuals and only the homozygotes carried an increased risk of disease development up to 3 times (p=0.05; OR= 3.15; 95% CI: 0.9275 to 10.6658) **(Table 12 and Figure 8)**.

Figure 8. POMP, SEMA6A and CACNA1A in XFS vs healthy subjects

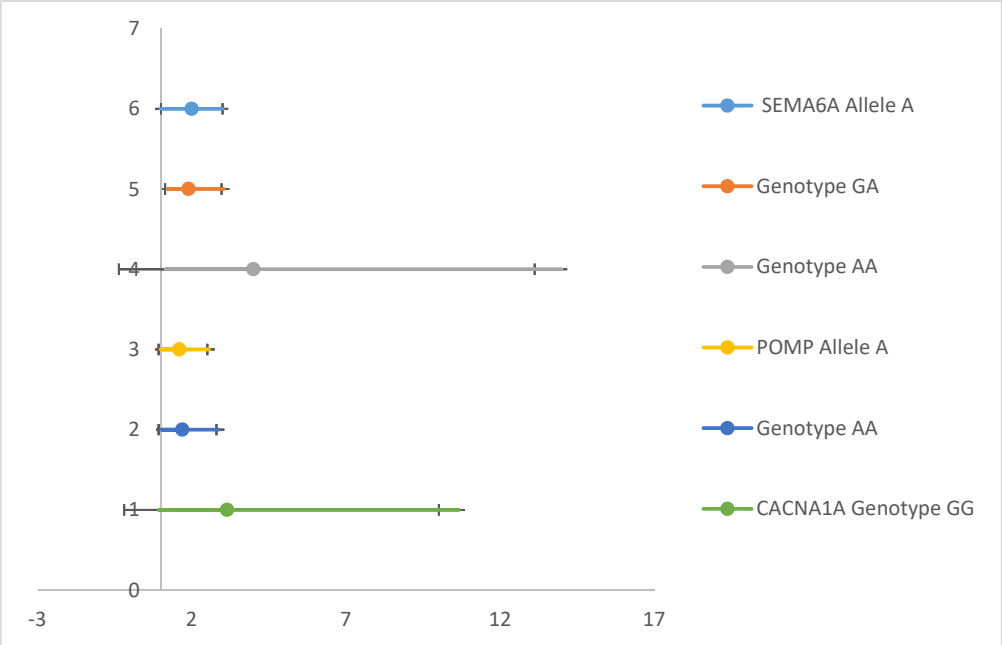


Table 12. Allele and Genotype Frequencies of POMP, SEMA6A and CACNA1A variants in XFS and healthy subjects

SNP		Controls % (n=199)	XFS % (n=132)	*P* value	OR (95% CI)
SEMA6A-rs10072088					
Allele	A	70.4	81.4	0.001	1.8 (1.2676 to 2.6973)
	G	29.7	18.6	0.001	0.5 (0.3299 to 0.8166)
Genotype	GA	42.3	32.5	0.002	1.9 (1.2246 to 3.0316)
	AA	49.2	65.2	0.004	4.0 (1.1531 to 13.9903)
	GG	8.5	2.3	0.004	0.2 (0.0715 to 0.8672)
	Total	98/84/17 (AA/GA/GG)	86/3/43 (AA/GA/GG)		
POMP-rs7329408					
Allele	A	9.8	14.8	0.04	1.6 (0.9931 to 2.5634)
	G	90.2	85.2	0.05	0.6 (0.3901 to 1.0069)
Genotype	AG	18.6	28	0.7	1.5 (0.0937 to 24.3786)
	AA	0.5	0.8	0.05	1.7 (1.0217 to 2.8713)
	GG	80.9	71.2	0.05	0.6 (0.3483 to 0.9788)
	Total	161/37/1 (GG/AG/AA)	94/37/1 (GG/AG/AA)		
CACNA1A-rs4926244					
Allele	G	21.21	18.34	0.4	1.2 (0.8122 to 1.7690)
	A	78.8	81.7	0.4	0.8 (0.5653 to 1.2313)
Genotype	AG	42.3	32.5	0.7	1.08 (0.6801 to 1.7043)
	GG	8.5	2.3	0.05	3.15 (0.9275 to 10.6658)
	AA	49.2	65.2	0.05	0.3 (0.0938 to 1.0782)
	Total	40/84/8 (AG/AA/GG)	65/130/4 (AG/AA/GG)		

For the other three genes tested (TMEM136 rs11827818, AGPAT1 rs3130283, RBMS3 rs12490863) we did not observe any statistically significant differences in allele frequencies. Previously reported high-risk alleles were present both in controls and

affected individuals in 10-15% of cases respectively and they did not confer any risk of disease development. The results are shown in **Table 13**.

Table 13. Allele and Genotype Frequencies of RBMS3, TMEM136 and AGPAT1 variants in XFS and healthy subjects

SNP		Controls % (n=199)	XFS % (n=132)	*P* value	OR (95% CI)
RBMS3-rs12490863					
Allele	A	9	10.7	**0.5**	1.2 (0.6797 to 1.9400)
	G	91	89.3	**0.5**	0.8 (0.5155 to 1.4712)
Genotype	GA	17.1	19.8	**0.5**	1.2 (0.6956 to 2.1274)
	AA	0.5	0.8	**0.8**	1.5 (0.6797 to 1.9400)
	GG	82.4	79.4	**0.8**	0.7 (0.4701 to 1.4376)
	Total	34/164/61 (GA/GG/AA)	104/26/1 (GA/GG/AA)		
TMEM136-rs11827818					
Allele	G	15.58	15.38	**0.9**	1.01 (0.6587 to 1.5637)
	A	84.4	84.6	**0.9**	0.9 (0.6395 to 1.5182)
Genotype	GA	27.1	27.7	**0.7**	1.004 (0.6175 to 1.6327)
	GG	2	1.5	**0.7**	1.3 (0.2370 to 7.2730)
	AA	70.9	70.8	**0.8**	0.7 (0.6125 to 1.6193)
	Total	54/141/4 (GA/GG/AA)	36/92/2 (GA/GG/AA)		
AGPAT1-rs3130283					
Allele	A	6.3	8.3	**0.3**	1.3 (0.7478 to 2.4600)
	C	93.7	91.7	**0.3**	0.7 (0.4065 to 1.3372)
Genotype	CA	12.6	16.7	**0.3**	1.3 (0.7352 to 2.5456)
	CC	87.4	83.3	**0.3**	0.7 (0.3928 to 1.360)
	AA	0	0		
	Total	54/141/4 (CA/CC/AA)	36/92/2 (CA/CC/AA)		

We then tested our results in a subgroup of exfoliation glaucoma cases. The frequencies of high-risk allele A of LOXL1 rs2165241 were significantly different when comparing cases and controls and associated with XFG (p=0.0002) and it did increase disease susceptibility to more than 4-fold (OR=4.5; 95% CI 1.7464 to 12.0506). It was present in 83% of affected individuals, almost 70% of them were homozygous and had 5.5-fold increased risk of XFS (*p*=0.0002; OR= 5.5; 95% CI: 1.8747 to 16.0444). Heterozygosity conferred 4.5-fold increased risk (*p*=0.0001; OR= 4.5; 95% CI: 2.8199 to 7.2454) compared to normal individuals.

For rs4886776 SNP G allele was the high-risk one. It was present in 84% of affected individuals. It conferred 3-times increased risk of XFG compared to normal controls (p=0.0001, OR=2.9; 95% CI 1.1815 to 7.0891). As with XFS we did not find statistically significant differences with heterozygotes, whereas in homozygotes, the risk of XFG was increased up to 6 times (*p*=0.003; OR=5.7; 95% CI 1.9934 to 16.7152).

As for rs8042039, G allele was present in all of the affected individuals and all of them were homozygous. **Figure 9 and Table 14** show the data.

Figure 9. LOXL1 SNPs in XFG vs healthy subjects

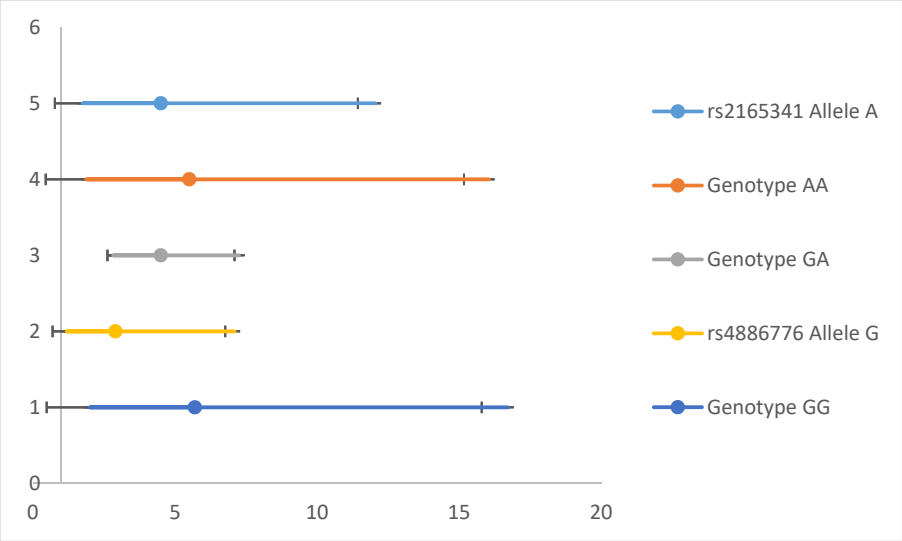


Table 14. Allele and Genotype Frequencies of LOXL1 SNPs in XFG vs healthy subjects

SNP		Controls % (n=199)	XFG % (n=18)	*P* value	OR (95% CI)
LOXL1 rs2165341					
Allele	A	58.5	83.3	0.0002	4.5 (1.7464 to 12.0506)
	G	41.5	16.7	0.0002	0.2 (0.0830 to 0.5726)
Genotype	AA	32.2	72.2	0.0002	5.5 (1.8747 to 16.044)
	GA	52.7	22.2	0.0002	4.5 (2.8199 to 7.2454)
	GG	15.1	5.6	0.0002	0.2 (0.0623 to 0.5334)
	Total	64/105/30 (AA/GA/GG)	13/4/1 (AA/GA/GG)		
LOXL1 rs4886776					
Allele	G	64.8	84.2	0.001	2.9 (1.1815 to 7.0891)
	A	35.2	15.8	0.001	0.3 (0.1216 to 0.3055)
Genotype	GG	32.7	73.7	0.003	5.7 (1.9934 to 16.7152)
	GA	64.3	21	0.5	1.8 (0.4846 to 3.2232)
	AA	3	5.3	0.0001	0.1 (0.0185-1.1778)
	Total	65/128/6 (GG/GA/AA)	14/28/1 (GG/GA/AA)		

Among other six genes studied, only SEMA6A and POMP were associated with XFG in our population. Allele frequencies of SEMA6A were significantly different when comparing cases and controls and associated with XFG (p=0.04). High-risk allele A increased disease susceptibility more than 3 times (p=0.04; OR= 3.4; 95% CI: 1.2676 to 2.6973). It was present in 86% of our patients, 65% of them were homozygous. Heterozygotes had twice increased risk (p=0.002; OR= 1.9; 95% CI:

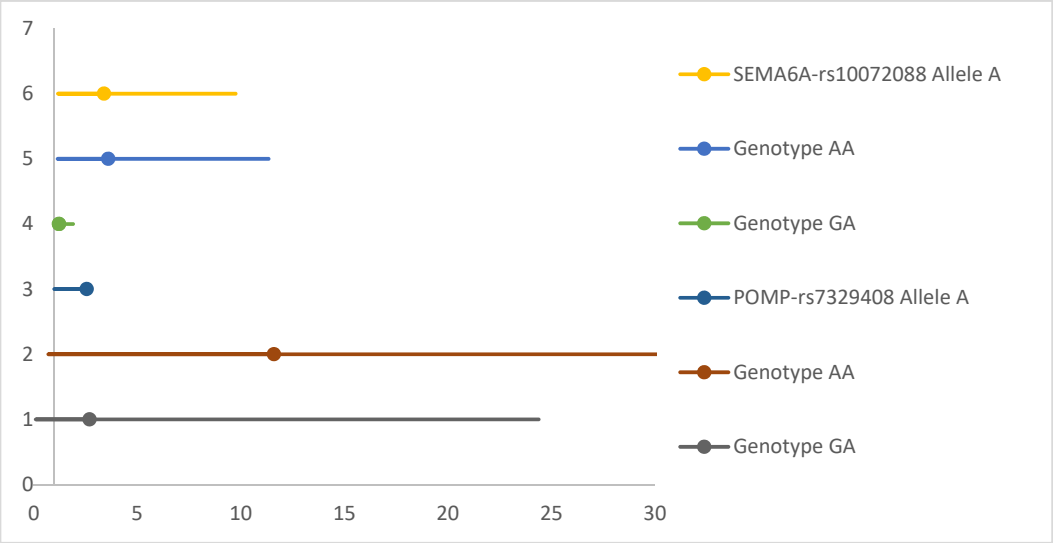
1.2246 to 3.0316), whereas, homozygotes had the risk of disease development up to 4 times higher (p=0.002; OR= 3.6; 95% CI: 1.1474 to 11.3402) compared to controls.

High-risk allele A of POMP was found in 22% of XFG cases. The likelihood of disease development rose up to 3 times in affected individuals (p=0.02; OR= 2.7; 95% CI: 0.9931 to 2.5634). Heterozygotes showed 3-fold increased XFG risk (p=0.05; OR= 2.7; 95% CI: 0.0937 to 24.3786) and homozygotes had up to 11 times (p=0.05; OR= 11.6; 95% CI: 0.6972 to 194.5768) higher risk when compared to individuals who were not carrying a high-risk allele **(Table 15 and Figure 10)**.

Table 15. Allele and Genotype Frequencies of SEMA6A and POMP genes in XFG and healthy subjects

SEMA6A-rs10072088					
Allele	A	70.4	86	0.04	3.4 (1.1663 to 9.7455)
	G	29.7	14	0.04	0.2(0.1026 to 0.8574)
Genotype	AA	49.2	65.2	0.002	3.6 (1.1474 to 11.3402)
	GA	42.3	32.5	0.002	1.9 (1.2246 to 3.0316)
	GG	8.5	2.3	0.004	0.2 (0.0715 to 0.8672)
	GG	8.5	2.3	0.004	0.2 (0.0715 to 0.8672)
	Total	98/84/17 (AA/GA/GG)	14/3/1 (AA/GA/GG)		
POMP-rs7329408					
Allele	A	9.8	22	0.02	2.7 (0.9931 to 2.5634)
	G	90.2	78	0.02	0.4 (0.3901 to 1.0069)
Genotype	AA	0.5	5.6	0.03	11.6 (0.6972 to 194.5768)
	GA	18.6	33.4	0.05	2.7 (0.0937 to 24.3786)
	GG	80.9	61	0.05	0.4 (0.3483 to 0.9788)
	Total	161/37/1 (GG/GA/AA)	11-06-01		

Figure 10. SEMA6A and POMP genes in XFG vs healthy subjects



For other genes studied we did not observe any statistically significant differencies between normal individuals and unaffected subjects. We also tested the data of XFG patients against XFS individuals but we did not find any gene to increase disease susceptibility. Though the XFG group was quite small and therefore could not reflect the whole picture.

Chapter 4. Discussion

Our study is the first attempt in Georgia characterizing the burden of XFS on cataract surgery. As we could clearly see, one fifth of all our cataract patients had small pupils and varying degrees of zonular laxity, one tenth of them had either deep or shallow anterior chambers, making surgery difficult. Of course, these conditions frequently coexist, further complicating the surgeon's task to safely and effectively perform the procedure.

There are studies characterizing these issues and complications among XFS patients. Scientists report a significant risk of zonular dialysis (OR 6.89), intraoperative miosis (OR 2.15), and lens luxation (OR, 9.49) in patients with XFS[100]. Our study showed even higher risks of these issues. According to Aravind XFS study, these patients are more likely to have a nuclear opalescence grade of more than 4 ($P = 0.001$), and to have a pupil size of less than 6 mm ($P < 0.001$) when compared with controls. The same authors report no statistically significant risk of intraoperative complication rates - 2.9% and 1.9% in the XFS and control groups, respectively ($P = 0.29$)[101], which is also consistent with our results.

There are also studies comparing the rates of posterior capsular tears during phacoemulsification in these two groups. Different studies report up to 6 times increased risks in XFS groups[102]. We did not find any significant association of posterior capsular tears with XFS. In our case the rates did not differ between two groups. The same authors improved outcomes in cases of early IOL implantations before removal of last quadrant of the nucleus, therefore protecting the posterior capsule[102].

Vitreous loss, one of the most severe complications of cataract surgery is reported in around 4% of surgical patients and this complication is related to XFS in most of them[103], also very similar to our findings.

Considering that more than a third of our cataract patients have so many special considerations intra- as well as postoperatively, it can be said that XFS definitely is a huge burden for anterior segment surgeons in our country. This fact indicates that surgeon experience is of utmost importance when dealing with XFS cases.

The detection of XFM in several visceral organs, such as lungs, gall bladder, liver, kidney, skin, meninges, heart and blood vessels, has led to hypothesis that XFS can be associated with increased risk of different systemic comorbidities, most importantly vascular ones. Some clinical studies have suggested these associations too, but others have not found any correlations. For example, according to the investigation of Citiric et al., the prevalence of coronary artery disease is significantly higher in patients with XFS than in normal individuals[68]. French at al. showed that the risk of IHD was 70% higher and the risk of MI was almost doubled in XFS patients compared to controls[69]. These results are very consistent with our findings. On the other hand, Tarkkanen et al. also evaluated IHD in glaucoma patients with or without XFS, finding no significant association[71]. No link between XFS and XFG to IHD has been found by the study from Emiroglu et al.,[70]. It should be noted though, that the age group evaluated by this study was significantly younger than ours, hence the results.

Many studies have also reported the association of XFS with cerebral ischemia. Yuksel et al. showed doubled prevalence of white matter ischemic areas in patients with XFS as well as XFG[74]. Akarsu reported significant reduction of transcranial Doppler parameters in XFS patients[104], while Rittland showed that XFG patients had significantly higher risk of acute cerebrovascular events as compared to normal controls[105]. These findings are very consistent with ours, reporting tripled risk of stroke/TIA in XFS patients.

The exact underlying pathogenic mechanisms of vascular comorbidities in XFS patients are not known, though several pathways have been suggested. XFM

accumulation around endothelial cells may disrupt their normal basement membranes and lead to impairment of their function. On the other hand, these deposits, together with increased concentration of a very powerful vasoconstrictor, endothelin-1, may lead to decreased elasticity of vascular walls and therefore, increased resistance[106]. Increased homocysteine levels, associated with XFS may also play the role in degradation of elastic structures of the vascular walls[107]. Moreover, oxidative stress markers have also shown to be increased in the aqueous humor, as well as in the serum of XFS patients. The imbalance of MMPs and their tissue inhibitors have been revealed by several investigators[60]. All these mechanisms may be associated with vascular comorbidities of XFS and most importantly, may lead to acute vascular events.

In contrast to other clinical studies, we did not observe an association between XFS and arterial hypertension but we did see the correlation with IHD and acute cardiovascular and cerebrovascular events. We did not have the opportunity to thoroughly investigate our cataract patients for other quantitative biochemical risk factors of these events. Therefore, our investigation was only epidemiological, suggesting, that XFS patients need closer attention and monitoring by general practitioners.

The hallmark of XFS is the accumulation of pathological fibrillo-granular material in different structures of the anterior segment of the eye. XFM is produced by many types of cells, such as ciliary epithelial cells, epithelial cells of the lens, trabecular and corneal endothelial cells and all cell types of the iris[108]. Interestingly, it is also produced by extraocular cells, such as fibrocytes, vascular cells, and muscle cells and is deposited in various organs like liver, brain, heart, lungs and skin[2], hence the association of XFS with systemic disorders, like transient ischemic attacks, stroke, myocardial infarction[2], atrial fibrillation, inguinal hernias and pelvic organ prolapse[109].

The fibrils are composed of highly glycosylated proteoglycan/glycoprotein complex which is very resistant to enzymatic cleavage[108]. Its amorphous protein core includes basement membrane components, like laminin and fibronectin, elastic fibers, such as fibrillin-1 and elastin. It also contains enzymatically active components, such as metalloproteinases, the extracellular chaperone clusterin[110], and the cross-linking enzyme LOXL1[108]. The letter is the key enzyme of elastogenesis and elastic fiber homeostasis. It is an extracellular copper-dependent amine oxidase that catalyses the first step in the formation of crosslinks in collagens and elastins. A highly conserved amino acid sequence at the C-terminus end possesses amine oxidase activity, whereas the N-terminus is poorly conserved and may have additional roles in developmental regulation, senescence, tumor suppression, cell growth control, and chemotaxis to each member of the family[111]. In early stages of the disease, increased synthesis of elastic fiber components coincides with upregulation of LOXL1, which participates in the abnormal cross-linking and misfolding of the newly synthesized extracellular matrix. This ultimately leads to the aggregation and accumulation of exfoliation deposits. Interestingly, in advanced stages of the disease, LOXL1 expression is downregulated, possibly by compensatory mechanisms, as the protein accumulates in the extracellular space[108].

It has been suggested that in XFS LOXL1 itself is misfolded and dysfunctional. Its N-terminus domain exists in a highly disordered state and a substantial amount of it is found to be processed for degradation by autophagy[112]. As shown in multiple studies, LOXL1 gene has several variants which are associated with XFS[99,113,114]. The proteins coded by these defective genes have multiple disorder probability domains[112] which supposedly result in misfolding of the enzyme with alteration of its function and resistance to cleavage.

In general, misfolded, denatured and damaged proteins are degraded by proteasomes and autophagy systems of the cells. If the synthesized protein is defective,

the first response is the upregulation of chaperones in attempt to fold the proteins properly[115]. If this mechanism fails, the protein is dislocated to the cytosol, where it undergoes ubiquitination, marking it for degradation by the proteasome. When the proteasome is dysfunctional, the damaged proteins are shuttled out of the cell. This mechanism appears to be protective in short-term, while in long-term, the result is a build-up of dysfunctional materials[116]. If the damaged proteins escape these two lines of defense, they become encapsulated into autophagosomes, eventually to be degraded by lysosomes[112].

It can be suggested that the combination of a defective synthesis of the LOXL1 protein resulting from LOXL1 gene variants and the inability to degrade LOXL1 containing protein aggregates produces XFM. Studies have demonstrated that XFS cells fail to properly transport lysosomes and autophagosomes to the perinuclear area, where autophagic clearance takes place[112]. Also, it has been shown that the clearance process is very slow in XFS[117]. The fact that LOXL1 is directed to lysosomes, suggests that previous defense lines are defective. This could mean that chaperones fail to refold it and some studies have found clusterin, the mediator of chaperones to be upregulated in XFS[110]. Also, supposedly, proteasomes fail to degrade LOXL1 and here the role of POMP mutation, found in XFS patients, comes into play[118].

POMP gene encodes proteasome maturation protein, a short-lived maturation factor required for proteasome biogenesis. It recruits newly synthesized subunits of the enzyme and targets them to endoplasmic reticulum for final assembly and maturation[119]. POMP-mediated mechanisms allow for efficient organization of the assembly process of this complex protein. Genetic defect in POMP protein supposedly, causes alteration of its function and loss of efficiency in proteolytic processes leading to XFM accumulation. Aung *et al.* recently reported that immunoblot analysis showed significant reduction of POMP protein in the iris and ciliary body specimens, obtained from XFS eyes in comparison to control eyes[120]

As discussed previously, next step in removing damaged proteins from the cells is autophagy, involving the formation of an isolation membrane around the protein which further forms the double membraned autophagosome, which then fuses with endosomes and lysosomes[121]. The fusion process is mediated by P/Q voltage-dependent calcium channels. These channels consist of several subunits, one of them being a conducting pore forming subunit α1, encoded by CACNA1A gene[96]. The studies, including ours, have shown that mutation of this gene is related to XFS susceptibility. The reason for that theoretically could be a decrease in fusion ability and thus in autophagic capacity, leading to XFM accumulation.

Experimental and clinical studies have demonstrated that aberrations in normal proteolytic processes in the cells contribute to the pathogenesis of several neurodegenerative disorders, which are associated with pathologic aggregation of proteinaceous materials and are collectively called aggregopathies. These include Alzheimer's disease[122], Parkinson's disease[123] and Huntington's disease[123]. These age-related aggregopathies show various degrees of genetic linkage to nonsynonymous single nucleotide polymorphisms, which result in a single amino acid substitution in a coded protein. This does not seem to largely affect protein function but may increase misfolding rates for the polypeptide chains during synthesis[112].

Previous electron microscopy studies on human XFS eyes showed the presence of high calcium concentration in direct association with aggregating XFS fibrils[124]. It has been demonstrated that calcium plays a role in maturation of a precursor fibrillin and in stabilization of fibrillin molecules and microfibrils[125]. It has also been shown that fibrillin is significantly more susceptible to proteolytic degradation in the absence of calcium[126]. Thus, it can be hypothesized that altered function of a calcium channel could lead to alterations of calcium concentrations that may facilitate the formation and stabilization of XFS aggregates.

In a recent study, the immunofluorescence microscopy analysis showed positive CACNA1A immunoreactivity in the different structures of the human eye. These included ciliary body, iris, anterior lens epithelium, optic nerve glia and vascular endothelial cells. In the retina, strong diffuse CACNA1A staining was seen in the photoreceptor inner segments (IS), inner nuclear layer (INL) and outer nuclear layer (ONL) and nerve fiber layer (NFL)[96]. On the other hand, the eyes affected by XFS showed only focal and patchy immunostaining of the IS, ONL, INL and NFL[96]. Interestingly, co-localization of CACNA1A and LOXL1 was observed only in the epithelium of the ciliary processes. The XFM in XFS eyes showed LOXL1 positive staining with negligible CACNA1A immunoreactivity[96]. These findings suggest that these proteins could contribute to disease development in different ways in different sites.

Nowadays, elevated IOP is considered only as a risk factor, not an etiologic one for glaucoma development, as normal tension glaucoma represents about 50% of glaucoma diagnoses[127]. It is well known that optic nerve atrophy still goes on despite lowering IOP[128]. These observations suggest that other reasons of neurodegeneration should be looked for.

In healthy neurons, Ca^{2+}-dependent processes influence different cellular functions, like generation and transmission of electrical signals, membrane trafficking, exocytosis and intracellular respiration. Calcium also activates a cascade of events that result in gene expression and that are essential for dendritic development, neuronal survival, and synaptic plasticity.

Calcium theory of neurodegenerative diseases has recently gained popularity. Defective intra- and extracellular calcium homeostasis, besides defects in autophagy, was observed in neurodegenerative disorders, like Alzheimer's disease[129], Parkinson's disease[130] and Huntington's disease[131]. Several neurodegenerative diseases have also

been linked to mutations of CACNA1A. These include spinocerebellar ataxia type 6, episodic ataxia type 2, and familial hemiplegic migraine with aura and transient hemiplegia[132]. An important mechanism of axonal loss neurodegenerative disorders is increased influx of extracellular Ca^{2+}, which triggers degradation of cytoskeleton through activation of intracellular enzymes, namely caspases. As mentioned previously, immunohistochemical studies have demonstrated CACNA1A staining in retinal cells, including nerve fiber layer. Theoretically, defective calcium homeostasis, associated with defects in this gene could be one of the factors, associated with neurodegeneration in glaucoma too.

Very interesting findings have recently been published by Shiga *et al*[133]. Using GWAS they identified new susceptibility loci for Primary Open Angle Glaucoma. Interestingly, seven loci, including LOXL1 were exclusively present in Japanese POAG patients as compared to other multiethnic groups. They excluded XFS from their cases, but found a significant association of LOXL1 rs1048661 with POAG susceptibility. The authors suggested that defects of elastin, a major component of extracellular matrix of the lamina cribrosa, could contribute to its structural deformity. It is well known that RGC axons are most vulnerable as they pass through the lamina cribrosa plates unmyelinated[134]. Therefore, their structural integrity could be compromised, leading to the development of glaucoma.

Our study showed significant susceptibility to XFS and XFG in patients carrying rs2165341, rs4886776 and rs8042039 variants of LOXL1. We also found increased risk of disease development in individuals, carrying CACNA1A rs4926244, POMP rs7329408 and SEMA6A rs10072088 genes. The rest of examined genes did not show any statistical significance when compared with normal controls. The possible ways these defective genes could contribute to XFS are described above.

The limitations of our study included very small group of XFG patients. Another limitation was the lack of long-term follow-up of XFS patients. This means that we do not know if any of these patients would develop XFG with time, therefore comparing a subgroup of XFG patients with XFS only individuals might not give correct results.

Conclusions

1. The prevalence of XFS is high and affects more than a third of Georgian patients undergoing cataract surgery.
2. XFG is encountered in 4.6% of Georgian patients undergoing cataract surgery.
3. Technical difficulties (small pupil, zonular laxity/dialysis) and complications (capsular bag loss, vitreous loss, aphakia with the need of IOL fixation) are encountered significantly more frequently during phacoemulsification in patients with XFS, necessitating advanced surgical skills. These issues are mainly seen in patients with higher grade cataracts.
4. Our study confirmed the results of other studies, showing the association of XFS with systemic vascular diseases: comorbidity is significantly more prevalent in cataract patients with XFS.
5. Increased risk of XFS is seen in carriers of LOXL1, CACNA1A, POMP, SEMA6A, SNPs, whereas TMEM136, RBMS3 and AGPAT1 SNPs are not associated with increased risk of this condition.
6. Increased risk of XFG is seen in carriers of LOXL1, POMP, SEMA6A SNPs, whereas CACNA1A, TMEM136, RBMS3 and AGPAT1 SNPs are not associated with increased risk of this condition.

Practical Recommendations

1. Given the high prevalence of XFS in Georgian patients, it is very important general ophthalmologists and cataract surgeons to determine adequate timing of phacoemulsification and recommend earlier interventions for safer surgery and better outcomes.
2. Due to the fact that delayed cataract surgery in XFS patients may potentially lead to severe complications, the health care and insurance systems should consider XFS as a relevant factor in determining the timing of intervention.
3. Cataract surgeons dealing with XFS patients are required to have advanced surgical skill in order to safely and effectively perform the procedures.
4. The epidemiology of XFS in our country indicates the need deeper learning of this topic in our residency and CME programs.
5. Given the complex genetic mechanisms of XFS and XFG development, which are not exactly determined to date, frequent IOP monitoring and optic nerve head evaluations should be performed by general ophthalmologists in order to prevent irreversible blindness in these patients.
6. The association of XFS with systemic vascular diseases found in our study, shows the need of early referral to general practitioners for closer monitoring.
7. Our research demonstrates the need of further studies in the field of XFS and systemic vascular diseases and the role of ophthalmologists in disease prevention and treatment.

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