Exfoliation syndrome: assembling the puzzle pieces

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ABSTRACT.

Purpose: To summarize various topics and the cutting edge approaches to refine XFS pathogenesis that were discussed at the 21st annual Glaucoma Foundation Think Tank meeting in New York City, Sept. 19–20, 2014.

Methods: The highlights of three categories of talks on cutting edge research in the field were summarized.

Results: Exfoliation syndrome (XFS) is a systemic disorder with a substantial ocular burden, including high rates of cataract, cataract surgery complications, glaucoma and retinal vein occlusion. New information about XFS is akin to puzzle pieces that do not quite join together to reveal a clear picture regarding how exfoliation material (XFM) forms.

Conclusion: Meeting participants concluded that it is unclear how the mild homocysteinemia seen in XFS might contribute to the disarrayed extracellular aggregates characteristic of this syndrome. Lysyl oxidase-like 1 (LOXL1) variants are unequivocally genetic risk factors for XFS but exactly how these variants contribute to the assembly of exfoliation material (XFM) remains unclear. Variants in a new genomic region, CACNA1A associated with XFS, may alter calcium concentrations at the cell surface and facilitate XFM formation but much more work is needed before we can place this new finding in proper context. It is hoped that various animal model and ex vivo systems will emerge that will allow for proper assembly of the puzzle pieces into a coherent picture of XFS pathogenesis. A clear understanding of XFS pathogenesis may lead to ‘upstream solutions’ to reduce the ocular morbidity produced by XFS.

Key words: animal models – CACNA1A – exfoliation syndrome – homocysteine – LOXL1

Introduction

Exfoliation syndrome (XFS) is the most common recognizable cause of open-angle glaucoma worldwide, accounting for the majority of cases in some countries (Ritch 1994). It is a public health problem because its ocular impact transcends open-angle glaucoma to also include angle closure glaucoma (Damji et al. 2009), a keratopathy that involves all corneal layers (Zheng et al. 2011), age related cataract (Puska & Tarkkanen 2009), a clinically significant zonulopathy (Naumann 1988; Davis et al. 2009), altered blood aqueous barrier integrity that can manifest as a pseudouveitis (Chern et al. 1994), and retinal venous occlusive disease (Prata et al. 2010; Ritch et al. 2010). Interestingly, XFS deposits are recapitulated in organs throughout the body (Schlotzer-Schrehardt et al. 1992).

While much is known about the clinical and histopathological features of XFS (Ritch & Schlotzer-Schrehardt 2001), many questions remain unanswered. For example what is the earliest manifestation of the disease at the ultrastructural and light microscopic levels? How is exfoliation material (XFM) assembled at the molecular level? Why is the disease asymmetric in a high percentage of cases? Could there be factors in the fellow eye that render it relatively impervious to XFM deposition? While XFM deposits occur in non-ocular tissue, one of the most frequently described systemic clinical finding is sensorineural hearing loss (Cahill et al. 2002; Shaban & Asfour 2004; Aydogan Ozkan et al. 2006; Turacli et al. 2007; Detorakis et al. 2008; Yazdani et al. 2008; Papadopoulos et al. 2010, 2012; Palibei et al. 2011); yet, most of these studies were not performed in conjunction with otologists, nor do they provide any otopathological correlation. The subject of sensorineural hearing loss in XFS deserves more carefully conducted study in broader populations. While XFM is located in many other organs (Schlotzer-Schrehardt et al. 1992), the clinical consequences of these deposits
are equivocal. For example, reports on an association with abdominal aortic aneurysm (Schumacher et al. 2001; Hietanen et al. 2002; Gonen et al. 2013) and cardiovascular disease (Citrik et al. 2007; Sekeroglu et al. 2008; Eminioglu et al. 2010; Demir et al. 2011) have been inconsistent and while studies regarding association with cerebrovascular disease exist (Mitchell et al. 1997), there is no evidence that XFS is associated with increased risk of cardiac or all-cause mortality (Ringvold et al. 1997; Shrum et al. 2000; Tarkkanen & Kivela 2014; Svensson & Ekstrom 2015). Discrepancies between reports of critical end-organ involvement and predicted relations to mortality could be the result of study methodology or due to the inconsequential amount of material deposited in extracellular tissues. There are new reports of several environmental exposures for XFS identified in population-based studies (Stein et al. 2011; Pasquale et al. 2012; Kang et al. 2014a,b), two genetic susceptibility markers (LOXL1 and CACN1A1) that may dysregulate extracellular matrix metabolism of XFS. Homocysteine (Hcy) is a non-protein amino acid that differs from its homologue, cysteine due to an additional CH₂ bridging group. Hcy levels are modestly, but consistently higher in serum, aqueous humor and tears of XFS patients compared to controls. Hcy is not particularly concentrated in the anterior segment, the most important site of ocular pathology in XFS (Table 1). This raises questions about whether this amino acid is a disease driver, disease biomarker or an innocent bystander to some biochemical disease driver, disease biomarker or an innocent bystander to some biochemical process related to Hcy metabolism.

If Hcy is a causative marker for XFS, then genetic conditions that produce Hcy levels comparable to those seen in XFS might be associated with the disease. The common functional C667T and A1298C polymorphisms in the methylentetrahydrofolate reductase (MTHFR) gene reduce enzymatic activity for catalysing the conversion of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, a co-substrate for homocysteine re-methylation to methionine producing mild hyperhomocysteinemia comparable to that seen in XFS (Frosst et al. 1995; Weisberg et al. 1998); yet, gene association studies fail to find an association between these polymorphisms and XFS (Fan et al. 2008; Xu et al. 2012). These data do not support genetic variation as the mechanism for homocystinuria or CBS deficiency is a rare autosomal recessive disorder and while these patients generally have a shortened life span, no association between this disorder and XFS has been reported. It is interesting to explore the effects of Hcy on ECM proteins, given that ECM metabolism is dysregulated in XFS. Hcy has a high acid dissociation constant (pKa = 10.0) relative to cysteine (pKa = 8.3) rendering it a highly reactive nucleophile that forms strong disulfide bonds (S-S) with cysteine (cys) residues in proteins. In fact the disulphide bonds formed by R-S-S-Hcy protein adducts are stronger than cys-S-S-cys bonds, allowing Hcy to attack disulphide bonds in proteins (Glushchenko & Jacobsen 2007). Thus, proteins with high cysteine content such as the fibrillins, and the associated microfibrillar proteins, namely the fibrillins and latent TGF beta binding protein-2 (LTBP-2) (Downing et al. 1996), are subject to trans sulphuration by high Hcy concentrations. Numerous mutations in the fibrillin-1 gene (FBNI) produce Marfan syndrome (Davis & Summers 2012) and the interaction between high Hcy concentrations and fibrillin-1 could produce similar phenotypes. Hcy concentrations of 300 μM completely abolish calcium binding in the calcium binding Epidermal Growth Factor domains in fibrillin-1, rendering the protein highly susceptible for proteolytic degradation (Hubmacher et al. 2005). Furthermore high Hcy levels reduce fibrillin-1 multimer formation and impair fibrillin self- interaction and assembly (Hubmacher et al. 2010). This discussion is highly relevant to the zonulopathy that occurs in homocystinuria and Marfan.

What is the Role of Homocysteine in Exfoliation Syndrome?

Homocysteine (Hcy) is a well-studied biomarker potentially related to XFS (Vessani et al. 2003; Bleich et al. 2004; Roedl et al. 2007; Xu et al. 2012). Hcy is a non-protein amino acid that differs from its homologue, cysteine due to a high acid dissociation constant (pKa = 10.0) relative to cysteine (pKa = 8.3) rendering it a highly reactive nucleophile that forms strong disulfide bonds (S-S) with cysteine (cys) residues in proteins. In fact the disulphide bonds formed by R-S-S-Hcy protein adducts are stronger than cys-S-S-cys bonds, allowing Hcy to attack disulphide bonds in proteins (Glushchenko & Jacobsen 2007). Thus, proteins with high cysteine content such as the fibrillins, and the associated microfibrillar proteins, namely the fibrillins and latent TGF beta binding protein-2 (LTBP-2) (Downing et al. 1996), are subject to trans sulphuration by high Hcy concentrations. Numerous mutations in the fibrillin-1 gene (FBNI) produce Marfan syndrome (Davis & Summers 2012) and the interaction between high Hcy concentrations and fibrillin-1 could produce similar phenotypes. Hcy concentrations of 300 μM completely abolish calcium binding in the calcium binding Epidermal Growth Factor domains in fibrillin-1, rendering the protein highly susceptible for proteolytic degradation (Hubmacher et al. 2005). Furthermore high Hcy levels reduce fibrillin-1 multimer formation and impair fibrillin self- interaction and assembly (Hubmacher et al. 2010). This discussion is highly relevant to the zonulopathy that occurs in homocystinuria and Marfan.

<table>
<thead>
<tr>
<th>Body fluid</th>
<th>Exfoliation syndrome (μM)</th>
<th>Controls (μM)</th>
<th>P-value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>15.5</td>
<td>11.8</td>
<td>0.012</td>
<td>Bleich et al. (2004)</td>
</tr>
<tr>
<td>Aqueous Humor</td>
<td>2.5</td>
<td>1.3</td>
<td>&lt;0.0001</td>
<td>Bleich et al. (2004)</td>
</tr>
<tr>
<td>Plasma</td>
<td>14.5</td>
<td>10.2</td>
<td>&lt;0.001</td>
<td>Roedl et al. (2007)</td>
</tr>
<tr>
<td>Tears</td>
<td>0.24</td>
<td>0.13</td>
<td>&lt;0.001</td>
<td>Roedl et al. (2007)</td>
</tr>
<tr>
<td>Plasma (meta-analysis of 14 studies)</td>
<td>10.1–20.3</td>
<td>8.4–17.4</td>
<td>&lt;0.001</td>
<td>Xu et al. (2012)</td>
</tr>
</tbody>
</table>

μM = micromolar.

Table 1. Total homocysteine levels in exfoliation syndrome versus controls.
Methionine - Homocysteine Cycle

Fig. 1. The methionine – homocysteine cycle contains re-methylation and transsulfuration components. The enzyme S-adenosyl homocysteine hydrodase (SAH) contributes to methylation of DNA and RNA. Other abbreviations: CBS = cystathionine-β-synthase; MTR = S-methyltetrahydrofolate-homocysteine methyltransferase; MTHFR = methyltetrahydrofolate reductase; THF-tetrahydrofolate; MTHF = 5-methyltetrahydrofolate.

Chambers et al., Circ. Res. (2001)

syndrome in contrast with XFS, since the zonule is composed predominately of fibrillin-1 (Hubmacher et al. 2014). In homocystinuria and Marfan syndrome the zonule is lax and elongates (Maumenee 1981), whereas in XFS the zonule is coated with XFM, frayed and focally disrupted. These data indicate that the shared phenotype features of Marfan syndrome and homocystinuria relate to the direct effect of fibrillin gene mutations in the former and the effect of Hcy modification on the gene product in the latter. The zonulopathy in XFS, on the other hand is probably unrelated to local Hcy levels and may result from abnormal lysosomal enzyme activity (Schlotzer-Schrehardt & Naumann 1994).

If Hcy is not a causative marker in XFS then exactly what is its role in disease pathogenesis? Vitamin B₆, vitamin B₁₂ and folic acid are important co-substrates for proper functioning of the methionine-Hcy cycle (Fig. 1). A synthesis of the literature suggests that serum folate levels are reduced in XFS patients while vitamin B₆ and vitamin B₁₂ are not (Xu et al. 2012). Furthermore, a large prospective dietary study in the U.S. indicated that health professionals were consuming more than adequate amounts of B₆ and B₁₂ but 50% were deficient in folate intake (Kang et al. 2014a). Furthermore, there was a trend towards reduced dietary folate intake (but not dietary B₆ and B₁₂) in association with XFG (Kang et al. 2014a). Thus, a chronically unbalanced methionine – Hcy cycle due to low dietary folate intake could be important in XFS. Chronic folate deficiency may prevent Hcy from cycling back to methionine, resulting in hypomethylation of key DNA loci (Fig. 1). The consequence is that local epigenetic mechanisms could cause altered LOXL1 expression and protein function, which could result in abnormal cross-linking of key macromolecules in XFM. Immunohistochemistry has shown LOXL1 as a component of XFM in early disease stages (Zenkel et al. 2011; Zenkel & Schlotzer-Schrehardt 2014). Another byproduct of imbalanced Hcy-methionine cycling is endothelial cell dysfunction, which could account for the increased retinal venous occlusive disease and other vascular findings seen in XFS (Hollo et al. 1998; Visontai et al. 2006, 2008; Prata et al. 2010; Ritch et al. 2010). It should be emphasized that the pieces of this complicated puzzle may require re-arranging as more genetic and serum biomarker data become available.

Genes, Epigenetics and Gene-Environment Interactions in Exfoliation Syndrome

Finding and characterizing genes that cause or contribute to XFS can define the underlying molecular events responsible for disease, suggest novel therapies targeting molecular pathophysiology and lead to the development of DNA-based diagnostic and screening tests. While XFS is readily diagnosed at the slit-lamp, a predictive model that incorporates genetic data creates an opportunity for preclinical diagnosis, allowing for more effective preventative intervention. A genome-wide association study performed in Iceland demonstrated that LOXL1 (lysyl oxidase like 1) is a major genetic risk factor for XFS (Thorleifsson et al. 2007). LOXL1 is a cross-linking enzyme necessary for elastin formation and maintenance. The association of LOXL1 with XFS has been replicated in populations throughout the world (Wang et al. 2014). Two missense variants in exon 1, R141L and G153D, were initially considered as the source of increased risk for disease in the locus. In fact the risk allele for the G153D conferred a 20-fold increased risk for XFS, an astounding high effect size for a common variant. However, while risk alleles were present in 99% of cases, they are also present in up to 80% of controls, suggesting that variants in this genomic region are necessary but not sufficient to produce disease. Furthermore, while the effect size for the G153D variant is high, the risk allele is flipped from A coding for glycine (labelled as G) to G coding for aspartate (labelled as D) in a South African population (Williams et al. 2010). The functionality of the exon 1 gene variants is not entirely clear, prompting researchers to explore the nearby promoter region and other 5' regulatory sequences for ‘causative’ variants related to XFS. The promoter region of LOXL1 is a particularly attractive candidate region to discover such variants because it contains many potentially active regulatory sequences that might influence gene expression including a LOXL1 antisense RNA, DNase hypersensitivity sites and DNA methylation regions. LOXL1 promoter
region haplotypes are also associated with XFS (Fan et al. 2011) but risk alleles in this region are also flipped in Asian populations (Dubey et al. 2014). The flipping of risk alleles in exon 1 and the LOXL1 promoter region suggests these variants are in linkage disequilibrium with yet other nearby loci that are important in XFS. Alternatively, the flipping of alleles at these various regions may signify that these are sites of local epigenetic methylation involved in regulating LOXL1 gene expression and risk for XFS.

Another issue to consider is that the frequency of LOXL1 risk alleles does not correlate with disease prevalence in various populations. XFS prevalence ranges from ~1% or less to >20% in various populations, yet the ratio of cases and controls with LOXL1 genetic susceptibility loci is relatively constant at ~95%:70%, depending on which locus one refers to (Pasquale et al. 2014).

While the search for other genetic loci associated with XFS continues, it does raise the possibility that the relation between LOXL1 gene variants is modified by environmental influences. From an epidemiological perspective, gene environment (GxE) interactions can be nonexistent (Figs 2–4) even if there are genetic and environmental influences that predict a trait of interest. Alternatively, genetic effects could be altered by the environment and these effects can be additive, multiplicative or of the cross-over variety (Figs 5 and 6, respectively). For one to entertain GxE interactions in XFS, one needs a cadre of environmental factors to consider for analysis. Work regarding the distribution of XFS as a function of geographical residence has revealed that colder ambient temperature and ultraviolet exposure are important environmental exposures in this disorder (Stein et al. 2011). Other possible environmental risk factors include high caffeine consumption (Pasquale et al. 2012) and low dietary folate intake (Kang et al. 2014a). While there is evidence for additive (Kang et al. 2010) and cross-over GxE interactions (Kang et al. 2011) in primary open-angle glaucoma, there is currently no evidence for GxE interactions in XFS.

A worldwide collaboration involving a genome-wide association study from Japan followed by two-stage replication using samples from 17 countries confirmed the association with LOXL1 and identified a novel locus involved in calcium signalling (CACA1A) (Aung et al. 2015). CACA1A codes for the transmembrane pore-forming subunit of the P/Q calcium channel, important for mediating calcium ion entry into excitable cells. It is also plays an important role in muscle contraction, hormone or neurotransmitter release and gene expression. The gene is expressed in ocular tissues relevant to XFS but is not found in XFM itself. High concentrations of calcium in XFM (Schlotzer-Schrehardt et al. 2001) has been reported and it is possible that alterations in cell surface calcium ions could facilitate the development of manifest disease. The connection between these observations, the CACA1A gene, and the pathogenesis of XFS is not yet known.

### Animal Models of Exfoliation Syndrome

A viable animal model of XFS could generate sufficient quantities of XFM for biochemical analysis. It has not been possible to generate XFM ex vivo and only small amounts can
be retrieved from surgical specimens or post-mortem material. Animal models would allow researchers to study the pathophysiology of XFS and how XFM forms. Such models could also confirm the role of environmental factors in influencing disease development and assess the efficacy of novel therapeutics to rescue the phenotype.

Models of XFS need not mimic all facets of the disease, but probably the aspect that is critically important to recapitulate is the ocular deposition of XFM. Model resources currently available are the *Loxl1* knock-out mouse (Wiggs et al. 2014) and the *Lyst* mouse (Trantow et al. 2009). Both models are intriguing but both have their shortcomings. The *Loxl1* (-/-) mouse develops cataract and has blood aqueous barrier dysfunction but there is no XFM, increased intraocular pressure (IOP) or optic neuropathy. The *Lyst* mouse (B6-*Lyst*<sup>B10</sup>) harbours a three base pair deletion that removes a single isoleucine from the carboxy terminus of the Lyst protein. *Lyst* codes for a lysosomal trafficking regulator and mutations in Lyst produce Chediak Higashi syndrome, a condition categorized by oculocutaneous albinism, immune system defects and abnormal clotting (Nagle et al. 1996). The mutation in these mice recapitulates the saw tooth iris transillumination defects seen in humans. There are XFM-like deposits but electron microscopy is required to see them. These mice also do not develop elevated IOP or glaucoma. *Cacna1a* null mice exhibit dystonia and cerebellar atrophy but the ocular phenotype is not known (Fletcher et al. 2001). Collectively these new models indicate that some *Loxl1* enzymatic is probably needed to generate XFM and that more research into the role of lysosomal function in XFS is needed.

**Future Directions in Exfoliation Syndrome Research**

To find the earliest clinical manifestation of XFS, it may be helpful to perform a mass screening of lens capsular specimens from patients with and without slit lamp biomicroscopic evidence of XFS according to an agreed upon experimental protocol that would involve immunohistochemistry and electron microscopy. Histological comparisons between pairs of human donor eyes from patients with unilateral or asymmetric disease might also provide novel insights into the pathogenesis of XFS.

The role of *Loxl1* gene variants in XFS requires more attention. Therefore, deep re-sequencing efforts are needed to identify the specific mutations in the *Loxl1* locus that confer risk for XFS. More work is needed to understand the cell biology of *Loxl1* in the eye. We know that LOXL1 orchestrates elastogenesis by binding its N terminus to fibulin 5. Perhaps CRISPR – caspase 9 technology can be used to cleave the N terminus of...
LOXL1 in the anterior segment to explore the phenotypic consequences. Other alternatives to learn about the cell biology of LOXL1 include the use of a rapidly aged, humanized drosophila model where the human LOXL1 gene variants can be inserted to study alterations in ocular phenotype. This approach has been used with success in Parkinson’s Disease (Siddique et al. 2014). Induced pluripotent stem (iPS) cells could also be used to model XFS and study LOXL1 biology. New growth factor cocktails produce better quality iPS cells and this approach has been used to model retinins pigmentation (Tucker et al. 2013). However, there are challenges for modelling XFS in this manner including the need to induce terminal differentiation of the eye cup into ciliary body and iris, as XFM has never been found in retinal tissue.

Most candidate genes, including candidate loci involved in the methionine-Hcy cycle are not associated with XFS. However, there is a suggestion that a locus in CLU may be associated with XFS (Krummiegel et al. 2009; Padhy et al. 2014). CLU encodes the protein clusterin, a chaperone highly expressed in the human iris that co-localizes with XFM (Doudevski et al. 2014). More work is needed to discover new XFS genes and this work is proceeding rapidly. Work in this area will require sample sizes on the order of 5000 – 10 000 cases to completely define the genetic architecture for XFS. The discovery of new XFS genes could spawn new XFS animal models that faithfully replicate the disease.

The future for assembling the puzzle pieces into a clear picture of XFS pathophysiology is bright. We need to look inside any cell that is destined to make XFM and understand how it is different from a normal cell. Exploration of extreme phenotypes, namely patients who develop the disease at a young age, may be helpful. The theme of case reports regarding young age of onset XFS suggests that cellular stress could play a critical role in XFM formation (Konstas et al. 1997; Amini et al. 2012). It will be important to incorporate cellular stress simulations in model systems of XFS moving forward. The long-term goal is to cure XFS by strategies that prevent or disassemble XFM at an early stage before ocular morbidity is realized.

References


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The Glaucoma Foundation held a meeting entitled, ‘Exfoliation Syndrome: Tying It All Together’ in New York, NY, September 19–20, 2014. The meeting organizers and moderators were R. Rand Allingham, MD (Professor of Ophthalmology, Duke University Medical Center, Durham, NC), Louis R. Pasquale, MD (Professor of Ophthalmology, Mass Eye and Ear Infirmary, Harvard Medical School, Boston, MA), Terete Borras, PhD (Professor of Ophthalmology, University of North Carolina, Chapel Hill, NC), John H. Fingert, MD, PhD (Associate Professor, Ophthalmology and Visual Science, University of Iowa, Iowa City, Iowa), Richard T. Libby, PhD (Associate Professor, Flum Eye Institute, University of Rochester School of Medicine, Rochester, NY), Janey L. Wiggs, MD, PhD (Professor of Ophthalmology, Mass Eye and Ear Infirmary, Harvard Medical School, Boston, MA), Barbara Wirostko, MD (Clinical Adjunct Associate Professor, Moran Eye Center, University of Utah School of Medicine, Salt Lake City, UT) and Robert Ritch, MD (Professor of Ophthalmology, Einhorn Clinical Research Center, New York Eye and Ear Infirmary of Mount Sinai, New York, NY).

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