Molecular genetics of familial hematuric diseases

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INTRODUCTION

The inherited hematuric diseases comprise a heterogeneous group of conditions, a common feature of which is microscopic hematuria (MH) during early childhood. Until recently, Alport Syndrome (AS) and thin basement membrane nephropathy (TBMN) were the only two known hereditary glomerular hematurias but this entity is now expanding through the recognition of additional genetic forms of glomerular hematuria. Hematuria may be a symptom of multiple disparate etiologies and therefore its thorough evaluation should include history, physical examination, urine analysis and radiological imaging. The evaluation should distinguish glomerular from nonglomerular origin and exclude prostatic and urinary infections, nephrolithiasis, polycystic kidneys, tumors or arteriovenous malformations. Additionally, hematuria may be the presenting sign of hypercalciuria and other familial and sporadic forms of urolithiasis. Patients with the painful loin
pain–hematuria syndrome, which still defies precise etiology and pathophysiology, also exhibit microhematuria [1, 2].

Currently for the practicing clinician, the following entities should come into mind when applying a differential diagnosis in a patient presenting with glomerular MH: MYH9 pathologies that are inherited by mutations in the MYH9 gene (non-muscle myosin heavy chain II A) and define a spectrum of rare autosomal-dominant macrothrombocytopenias. There are four clinical entities: the May–Hegglin anomaly, the Fechtner, Sebastian, and Epstein syndromes, sharing ultrastructural features with AS and associated with sensorineural deafness [3, 4] (Table 1). MH may also be the presenting sign of IgA nephropathy (IgAN), a frequent clinical condition which is the commonest cause of episodic macroscopic hematuria. In the overwhelming majority of cases, IgAN is not hereditary.

In this review, we shall concentrate on the inherited familial hematurias of glomerular origin, as we know them based on recent discoveries and genetics advancements, which implicate mainly three types of molecules, namely collagen IV (COL4) for AS and TBMN, CFHR5 of the alternative pathway of complement system which is mutated in C3/CFHR5 glomerulonephritis and fibronectin 1 (FN1), which is mutated in glomerulopathy with fibronectin deposits (GFND).

**MOLECULAR BIOLOGY OF FN1 AND GFND**

FN1 is a dimeric glycoprotein with multiple functions including a structural role and a role in wound healing, found in a soluble form circulating in plasma, as well as an insoluble, fibrillar form which is a constituent of the extracellular matrix. It interacts with specific integrin receptors on cell membranes, and it forms fibrils in the extracellular matrix, interacting with other glomerular basement membrane (GBM) components such as collagen IV and integrins. Distinct regions of the protein have been identified that also react with heparin (Hep-I-III). It is encoded by the FN1 gene on chromosome 2q35, and it encompasses 46 exons encoding 2,477 amino acids. In its monomeric form, the protein has a modular structure with type I, II and III repeats while the gene undergoes alternative splicing in three positions, giving rise to several protein isoforms.

FN1 mutations impair the assembly of protein dimers into fibrils and the balance between soluble and insoluble fibronectin, leading to its deposition in the glomerulus. The resulting disease, termed GFND, is inherited as an autosomal-dominant nephropathy characterized by MH, proteinuria and hypertension while patients progress to end-stage kidney disease (ESKD) during the second to sixth decade of life. This striking age-dependent penetrance may be attributed to the allelic heterogeneity or to the contributing role of unknown genetic modifiers or to both. It is associated with enlarged glomeruli and formation of fibronectin deposits in the mesangium and the subendothelial space. Positive immune reactivity for FN in the glomerulus is a pathognomonic feature of GFND [5].

Recent work [6] described 15 families with GFND. Three mutations were found in six families in the FN1 gene at 2q35. Two mutations (p.W1925R, p.L1974R) located in repeat I13 in the Hep-II heparin-binding domain were tested in vitro and were shown to reduce binding to heparin and to endothelial cells. Additional experiments showed impaired cell spreading and cytoskeleton, and were hypothesized to result in abnormal protein trafficking. Interestingly, a third mutation (p.Y973C) within the I14 repeat in the Hep-III domain was common in four families, one each from Italy, The Netherlands, Germany and Japan [6]. Despite its presence in families with disparate ethnic origin, haplotype analysis did not support a founder phenomenon. Even though GFND appears to be a rare disorder, it is reasonable to hypothesize that the elucidation of its molecular etiology may lead to the recognition of more families that present with MH and/or proteinuria where other more common causes are excluded.

**BIOCHEMISTRY AND MOLECULAR BIOLOGY OF COLLAGEN IV**

Collagens comprise a large family of 28 members, expressed in connective tissues throughout the body or as transmembrane proteins, with a broad range of functions including scaffolding, cell adhesion and morphogenesis [7]. In their mature form they function as triple-helical structures of homo- or heterotrimers. Every chain contains a typical collagenous amino acid sequence of repeating Gly-X-Y triplets, where X and Y are frequently prolines and 4-hydroxyprolines, contributing to the thermal stability. Glycine, as the smallest amino acid, is the only one that can fit at the center of the triple helix where the chains meet. Usually, most substitutions of glycines result in pathological situations, because of space constraints when substituted by bulkier residues [8]. Many collagens form long rod-like structures that also contain globular domains at the amino- and carboxy-terminus. Chain recognition and association occurs through their NC1 globular domains at the C-terminus and triple-helix forms by zippering towards the N-terminus. Some of them are fibrillar, forming nucleated fibrils by self-polymerization that act as cement in gluing together the extracellular milieu, interacting with other components and with cells. Prime examples of fibrillar collagens are type I, II, III and V. Type IV collagen does not form fibrils but a network after it is secreted in the extracellular space. Two collagen IV triple-helical protomers interact through their C-terminal NC1 domains while four protomers interact through their N-terminal 7S domains. There are six genes encoding for alpha chains that participate in collagen IV trimer formations, α1–α6, located in pairs in a head-to-head orientation, α1–α2 on chromosome 13q34, α3–α4 on chromosome 2q36.3 and α5–α6 on chromosome Xq22.3 (Table 1). Only three trimer combinations are biochemically permissible and found in nature, α1α1α2, α3α4α5 and α5α5α6, with distinct tissue distribution. In the glomerulus, initially there is α1α1α2, subsequently substituted by the α3α4α5 configuration during nephron maturation. The Bowman capsule and the skin basement membrane maintain trimers of α5α5α6 configuration. In contrast to the fibrillar collagens, collagen IV chains contain interruptions of the collagenous sequence, 22–26 depending on the chain, which are necessary for conferring
<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Major diseases</th>
<th>Selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1(IV)</td>
<td>COL4A1</td>
<td>13q34</td>
<td>Porencephaly; brain small-vessel disease with hemorrhage; autosomal-dominant hereditary angiopathy with nephropathy, including microscopic hematuria, aneurysms, and muscle cramps (HANAC syndrome).</td>
<td>[89–91]</td>
</tr>
<tr>
<td>α2(IV)</td>
<td>COL4A2</td>
<td>13q34</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>α3(IV)</td>
<td>COL4A3</td>
<td>2q36.3</td>
<td>Autosomal recessive Alport syndrome; autosomal-dominant Alport syndrome; benign familial hematuria and thin basement membrane nephropathy; thin basement membrane nephropathy associated with focal segmental glomerulosclerosis and chronic or end-stage kidney disease usually after 50 years.</td>
<td>[49, 92–94]</td>
</tr>
<tr>
<td>α4(IV)</td>
<td>COL4A4</td>
<td>2q36.3</td>
<td>Autosomal recessive Alport syndrome; autosomal-dominant Alport syndrome; benign familial hematuria and thin basement membrane nephropathy; thin basement membrane nephropathy associated with focal segmental glomerulosclerosis and chronic or end-stage kidney disease usually after 50 years.</td>
<td>[49, 92, 95]</td>
</tr>
<tr>
<td>α5(IV)</td>
<td>COL4A5</td>
<td>Xq22.3</td>
<td>Classic X-linked Alport syndrome with added proteinuria, progressive glomerulonephritis with chronic/end-stage kidney disease, characteristic alternating thinning/thickening or splitting of the basement membrane. Milder forms with later age at onset have been described; X-linked Alport syndrome with diffuse leiomyomatosis (contiguous gene syndrome); Alport syndrome, mental retardation, midface hypoplasia and elliptocytosis (AMME complex) resulting from a telomeric deletion of Xq22.3 which includes COL4A5 and additional genes.</td>
<td>[96, 97]</td>
</tr>
<tr>
<td>α6(IV)</td>
<td>COL4A6</td>
<td>Xq22.3</td>
<td>X-linked Alport syndrome with diffuse leiomyomatosis (contiguous gene syndrome).</td>
<td>[98]</td>
</tr>
<tr>
<td>Complement Factor H-related 5</td>
<td>CFHR5</td>
<td>1q32</td>
<td>Autosomal-dominant C3 glomerulonephritis with isolated complement C3 deposits, in the absence of immune complexes, progressing to proteinuria and chronic/end-stage kidney disease, usually after 50 years.</td>
<td>[76, 77]</td>
</tr>
<tr>
<td>Nonmuscle myosin heavy chain IIA</td>
<td>MYH9</td>
<td>22q11.2</td>
<td>Autosomal-dominant May–Hegglin anomaly, Sebastian, Fechtner and Epstein syndromes with macrothrombocytopenias and combinations of symptoms on a continuous spectrum including basement membrane thickening and lamellation, chronic/end-stage kidney disease, sensorineural deafness, cataracts. Hematological features include giant platelets and in the case of Fechtner syndrome granulocyte inclusions.</td>
<td>[4, 99, 100]</td>
</tr>
<tr>
<td>Fibronectin 1</td>
<td>FN1</td>
<td>2q35</td>
<td>Autosomal-dominant glomerulopathy with massive glomerular deposits of fibronectin (age-related penetrance, end-stage kidney disease progression).</td>
<td>[6]</td>
</tr>
</tbody>
</table>
flexibility to the network formed. Collagen IV is the most abundant molecule in the GBM, along with laminin, perlecain and other proteoglycans [9].

Type IV collagen is only found in basement membranes, including the GBM, which is one of the three crucial components of the glomerular filtration barrier (GBF), the other two being the fenestrated capillary endothelium on the inside and the podocytes outside. The podocyte foot processes bridge and form important cell tight junctions, the highly structured slit diaphragms with nephrin, nephrin-like proteins, podocin and others. Defects in some components of the GBF can result in hematuria and/or proteinuria and progress to chronic renal failure.

COLLAGEN IV NEPHROPATIES

Histology and genetics of AS

COL4 nephropathies include inherited familial glomerulonephritides that are caused by mutations in one of the genes encoding the α-chains of collagen IV molecules found in mature glomeruli, namely α3α4α5. AS is the classical hemorrhagic nephritis that was initially recognized by Arthur C. Alport to be inherited as a dominant disorder in the presence of nervous deafness [10]. Subsequently, it became clear that about 85% of the cases follow an X-linked inheritance (XLAS) because of mutations in the COL4A5 gene and the rest follow an autosomal recessive inheritance (ARAS) with homozygous/compound heterozygous mutations in either the COL4A3 or the COL4A4 gene. Some patients have been diagnosed with autosomal-dominant AS because they were heterozygous for mutations in COL4A3/COL4A4 [11–14]. Several years after the elucidation of the AS genetics, a growing number of patients have been reported who present with TBMN and progress to ESKD but have not always been regarded as suffering from autosomal-dominant AS (see below). It should be clarified that patients with TBMN due to heterozygous mutations in the COL4A3/COL4A4 essentially represent the carrier state of ARAS (see also [15]).

AS is a rare disease. The incidence in the general population remains unknown; however in the USA it is estimated to be 3% among children and 0.2% among adults with ESKD. In Europe, children on renal replacement therapy because of AS were estimated to account for 1.5%. An older study in Utah estimated a prevalence of 1/5,000 and a Finnish study estimated the incidence of AS to 0.2/10,000 live births [16–18].

AS patients invariably present with MH since childhood; episodes of macroscopic hematuria can also occur. Progressively they develop proteinuria and chronic kidney disease (CKD) and they reach ESKD usually during the second or third decade. The most frequent extrarenal features are sensorineural hearing loss in about 82.5% and ocular defects (dot and fleck retinopathy and anterior lenticulon) in about 44% of males with XLAS. The characteristic histological finding is alternate thinning and thickening of the GBM, splitting and lamellation of the lamina densa, while a basket weave appearance is also pathognomonic at electron microscopy (EM), accompanied by podocyte foot process effacement, which annuls the slit diaphragm. Focal and segmental glomerulosclerosis is seen with light microscopy. While nearly all female carriers will manifest MH, some will also progress to CKD at later ages and even ESKD (15% by age 60 years) owing to the underlying random X-chromosome inactivation, thus resulting in somatic cell mosaicism, which may unluckily inactivate preferentially the healthy gene. It is reported that women carriers of X-linked COL4A5 mutations will develop hematuria, proteinuria, hearing loss and ocular defects in proportions of 95, 75, 28 and 15%, respectively [19–21]. Even though EM is an indispensable tool in the diagnosis of glomerulonephropathies, an early biopsy in a young hematuric male which shows GBM thinning may not be adequate to differentiate between AS and TBMN [22–25]. The GBM width depends on gender and age, reaching its final width during adolescence and ranging between 300 and 400 nm, depending also on the normal range established by individual laboratories. Genetic testing has emerged as the gold standard in establishing the diagnosis of COL4 nephropathies, although the considerable length of COL4 genes that are encoded in 52, 48 or 53 exons for the α3, α4 or α5 chains, respectively, is still a challenge for most diagnostic laboratories. However, improvement in technology and lowering of the reagent costs prompted us and others to resolve to direct DNA analysis of genomic sequences, in an exon by exon approach. Next-generation sequencing approaches that apply massive parallel sequencing will facilitate the investigations, especially in cases of genetic heterogeneity exemplified by AS and ARAS [26]. Also, although the EM may reveal ultrastructural abnormalities reminiscent of Alport-like nephritis, it is not possible to differentiate between the molecular pathologies of the X-linked COL4A5 and the autosomal COL4A3/A4, something that is of paramount importance for family planning and counseling. The drawing of a detailed pedigree is essential for several reasons, and one might argue that the inheritance pattern will guide us for X-linked or autosomal inheritance; however, many times either the lack of adequate information in previous generations or the structure of the specific pedigree in combination with its small size, may not reveal the precise inheritance mode. The tissue distribution and trimer composition of the different collagen IV molecules is well known, and in most cases, immunohistochemical staining for the alpha-3 and alpha-5 chains will be pathognomonic. In XLAS, most causative mutations in α5-chain prevent normal triple-helix formation and secretion and lead to absence of the respective collagen IV chains from the extracellular matrix and result in negative staining for both the α3 and α5-chains in the GBM, as well as the Bowman’s capsule. Similarly, in cases of COL4A5 mutations, a skin biopsy may be diagnostic due to the absence of the encoded α5-chain [23]. To the contrary, in cases of ARAS there is absence of staining for α3 and α5-chains in the GBM (α2α3α5 composition), but the α5 staining is still normal in the Bowman’s capsule and the skin basement membrane where the collagen IV trimer has the α5α5α6 composition. However, a positive skin staining will not always differentiate with absolute certainty between autosomal and X-linked inheritance, as there are rare cases of mild X-linked disease which retain positivity for the α5-chain [27, 28]. Interestingly, recent results
indicated that maintained glomerular expression of the \( \text{COL4A3} \) chain is an early positive prognostic marker in patients with XLAS [29]. All in all, a quick and robust molecular investigation may resolve these uncertainties in combination with clinical data and a patient or a family history, perhaps averting the need for an invasive biopsy.

**Phenotypic heterogeneity-allelic heterogeneity**

With the study of hundreds of AS patients thus far, a juvenile and an adult onset of ESKD have been recognized. Even though several attempts were made to achieve genotype–phenotype correlation and some general guidelines have been derived, the outcome is still not satisfactory [19, 30, 31]. Notably, there have been no extensive biochemical studies to include the conserved glycines or other missense mutations within the triple-helical domain. Kobayashi et al. [32] investigated mostly the role of NC1 domain mutations which interfere with chain recognition and assembly and consequently with trimer formation and efficient secretion. Several large studies in Europe, the USA and China described the spectrum of clinical phenotypes and attempts were made to associate the genotype to the phenotype, considering more than 500 mutations reported in the \( \text{COL4A5} \) and more than 100 mutations in the \( \text{COL4A3A4} \) genes [19, 30, 33–35]. These studies suggested that the position and type of each mutation may dictate to some extent the severity of XLAS and how soon ESKD may ensue. However, as most mutations are missense changes due to single nucleotide substitutions, no correlation could be demonstrated between the position of a missense mutation and the age at ESKD. They did, however, observe that missense mutations were associated with the best prognosis, with an average age of 37 years old at onset of ESKD. Reasonably, the most severe mutations are those resulting in null synthesis of mature trimers or no secretion of trimers in the extracellular matrix, as evidenced by negative immunohistochemical staining. Milder mutations are those which apparently are hypomorphic, leading to synthesis of mature collagen IV trimers that are secreted, perhaps less effectively and are partially active in network formation. Perhaps owing to the nonfibrillar nature of this molecule, no dominant negative effects are experienced, as it largely happens with fibrillar collagens, which result in aberrant fibril formation and severe disease. This is because the association in partnership of normal with mutant molecules is suicidal as the mature trimer or the fibril formation is affected in its entirety. Despite the usefulness of these studies, it is still not possible for every specific mutation to conclude with absolute certainty on the prognosis for the patient before us. Among many, exceptions not conforming to these rules are mutations p.G624D and p.F222C, which, contrary to the predictions, are associated with mild and severe disease, respectively [36, 37]. Also, mutations in the NC1 domain that were anticipated to cause severe XLAS have been found in mildly affected patients, apparently because they do not always entirely prevent the chain assembly and the triple-helical formation [27, 38]. In a recent report, Tsiakkis et al. [8] showed that the single significant element in glycin substitutions associated with disease severity has been the number of carbons in the bulkier substituting residue, thus interfering with the triple-helical structure of collagens.

As regards the ARAS, there are fewer comprehensive reports on a large number of patients. According to European studies, 73% of patients reach ESKD or renal failure while hearing loss manifests in 77–91% and ocular changes in 61–91% [39, 40]. In a Chinese series of 17 patients, none had reached ESKD and the prevalence of hearing loss, and ocular abnormalities was much lower at 58 and 10%, respectively, most probably because all patients were still below the age of 18 years [41]. Reasonably, frameshift and truncating mutations were associated with more severe phenotypes but there is no detailed correlation.

**Mild X-linked AS and hypomorphic \( \text{COL5A5} \) missense mutations**

The molecular genetics approach enabled the study of families segregating \( \text{COL4A5} \) mutations with milder symptomatology, where AS had not always entered the differential diagnosis from the beginning [34, 38, 42–45]. Apparently, these mutations are hypomorphic and the mature collagen IV protomers maintain substantial residual activity in the GBM, thereby not presenting with the classical textbook picture as regards the clinical semiology and the ultrastructural appearance of the GBM. Rather, these patients present as phenocopies of TBMN, some of whom progress to CKD and ESKD at ages much later than anticipated for classical AS. Several such mutations have been described. One mutation with translational distribution is p.G624D, in exon 25 [34, 37, 44, 46]. We had diagnosed one Hellenic family carrying mutation p.G624D. The proband had presented at age 46 with persistent MH, proteinuria 3.3 g/day, hypertension and CKD. Without a revealing family history he reached ESKD at 50 years, with an EM ultrastructural appearance that did not exclude AS [37]. Further screening identified this mutation in six Hellenic families with 12 hemizygous males, only four of whom have reached ESKD at ages 61, 51, 50 and 39. Among all patients, there is complete absence of ocular signs and only two showed late onset sensorineural deafness. Interestingly, four families of Slovenian origin had been reported to segregate this mutation, diagnosed with benign familial hematuria [34]. Another similar mutation is the \( \text{COL4A5}-\text{p.P628L} \), identified in two large Cypriot families where overlapping symptoms in 9M/9F with microhematuria and proteinuria prevented the clear-cut recognition of the X-linked inheritance. EM showed that two patients had well-preserved glomeruli with widespread thinning of the GBM in adulthood, thereby supporting a likely diagnosis of TBMN [47–49]. Seven of nine affected males reached ESKD at ages ranging from 30 to 56 years. The remaining two males are currently 51- and 57-years old and only show mild renal insufficiency, with no extrarenal manifestations.

The milder presentation of patients carrying mutations p.G624D and p.P628L can be attributed to their exact position. p.G624D is in the 12th collagenous natural interruption of the triple-helical domain, converting a GIG interruption to a G4G one, perhaps not destabilizing very drastically the triple-helix compared with a similar substitution elsewhere in the collagenous domain. Similarly, p.P628L results in a new triplet,
Gly-Pro-Leu, substituting the first Y-position proline after the 12th natural collagenous interruption [37, 50]. This might destabilize less the triple-helix flanking the interruption, thereby reducing significantly the flexibility of the protomer and the resulting network [51]. In addition, it can be hypothesized that some mutations such as these near natural interruptions, do not drastically disrupt the zipper-like formation of the triple helix, which starts at the C-terminal NC1 domain, or interfere less with the binding of putative ligands in the matrix milieu. At the same time, it should be mentioned that not all natural interruptions are the same as another substitution in the second natural interruption, COL4A5-p.F222C, results in a severe glomerulopathy, distinct from Alport nephritis [36].

For completion, it should be mentioned that contrary to the general rules, several other mutations have been published for families with mild XLAS [42, 44–46]. Mutations, p.C1564S, p.L1649R, p.R1677Q, were found in the USA spread across several states as founder effects. One more recent article reported on a family of New Zealand origin, where three of eight males carrying mutation p.C1638Y progressed to ESKD at 26, 28, 40 years with no extrarenal symptoms. Five patients at 36, 39, 39, 46 and 72 years have only CKD [38]. It is interesting that all four mutations are located on the NC1 domain, presumably interfering with chain assembly. However, apparently once the assembly is achieved perhaps with some delay in the endoplasmic reticulum, the protomers are secreted and take part in network formation, resulting in milder phenotypes.

Overall, the recent literature from several laboratories around the world is sending a strong message that in addition to the classical progressive hereditary nephritis of AS, there are also mutations in the COL4A5 gene that present as phenocopies of TBMN. In cases of unclear mode of inheritance and inconclusive ultrastructural and clinical data, a molecular investigation may save the situation, including the analysis of all three COL4 genes. In view of this, it should come as no surprise if many more patients with milder mutations exist that are either undiagnosed or even worse, misdiagnosed. Finally, there is undoubtedly interfamilial, and most importantly intrafamilial, clinical heterogeneity with variable expression of the disease, as exemplified by the age of onset of ESKD or by the presence or absence of other extrarenal manifestations. One explanation is the role of epistatically acting modifier genes although none such modifier gene has been identified with certainty thus far for AS in humans. Several such loci have been implicated and mapped in animal models but none has been cloned [52–54].

**Thin basement membrane nephropathy**

Worldwide data suggest that about 1% of the population may have MH and thin basement membranes, a frequency that unavoidably leads to occasional superimposition of TBMN with other glomerulopathies. Importantly, 35–50% of IgAN patients do have GBM abnormalities, most frequently thin basement membranes, although it is not normally suggested that these patients be further investigated for a COL4 nephropathy [23]. Familiar MH can be the result of TBMN which is inherited as an autosomal-dominant disease, while previous publications estimated that 40–50% of patients with TBMN carry heterozygous mutations in the COL4A3/A4 genes. Based on our own experience with a large cohort of more than 250 patients with clinical data carrying COL4 mutations, there is incomplete penetrance of 5–10%. It is more than certain that there are more genes responsible for familial hematuria, as in our collection alone, we have microhematuric families that do not map to the chromosome 2q36.3 locus (Deltas and Pierides, unpublished results, and [55]). This condition used to be synonymous with benign familial hematuria that was accompanied by excellent prognosis on long follow-up. However, meticulous search of the older literature revealed that there where sporadic reports of small number of patients alluding to the fact that occasionally patients with TBMN expressed more serious phenotype, including CKD and even ESKD. Rogers et al. [56], in a 1973 clinical note, had stated that: The abnormality causing the hematuria can be called “benign” only after prolonged observation over a period of years with neither further morbidity nor mortality. One of the explanations for this adverse development was the probable co-inheritance of another glomerulopathy, perhaps IgAN, focal segmental glomerulosclerosis, minimal change disease, mesangioproliferative glomerulonephritis or others, something that cannot be excluded entirely considering the fairly high estimated prevalence of TBMN. This combination of pathologies could explain why some patients presented with isolated MH as a result of TBMN and on long follow-up they progressed to proteinuria, hypertension and CKD/ESKD. At the same time though, it was obvious that TBMN itself appeared to predispose some patients to a more adverse outcome, not conforming to the benign prognosis and rendering the term ‘benign familial hematuria’ as a misnomer [14, 57–61]. We believe that the term benign familial hematuria should be avoided because it is misleading and it only creates confusion.

A few years ago, we started studying a series of 12 families that we thought were segregating primary autosomal-dominant FSGS, based on renal biopsies from 15 patients. Our failure to detect causative mutations on relevant genes, ACTN4, CD2AP and TRPC6, prompted us to concentrate on another trait which segregated in all families, that of MH. DNA linkage analysis indicated linkage to 2q36.3 and re-sequencing of the COL4A3/A4 genes identified causative mutations, some of them being the result of strong founder effects. The presence of thin basement membranes established the dual diagnosis of FSGS and TBMN, clearly allowing the reasonable hypothesis that TBMN may predispose some patients to a more adverse outcome. Our more recent work, which included 213 patients in 22 families with TBMN and documented COL4 mutations in heterozygosity, revealed that they fit on a broad continuum of symptoms. The course of the disease appears to progress from isolated MH to additional proteinuria usually after the age of 30 years and subsequently to hypertension and CKD after the age of 50 years. In a cohort of 213 patients, more than 10% across all ages and 18.3% of patients older than 50 years reached ESKD. Interestingly, 26.6% of all patients and 48.1% of those over 50 years developed CKD of variable degree. Kaplan–Meier analysis shows...
that about 30% will develop ESKD by the age of 70 years (Figure 1A–C). Equally interesting is the observation that about 20% of patients reach advanced ages with only isolated MH or low-grade proteinuria. Among all of the patients in this cohort, there are 155 who carry the same mutation, COL4A3-p.G1334E, which is identical-by-descent and evidently it is the result of a founder dating 250–300 years back [62]. This makes it even more interesting in view of the indisputable finding that even among carriers of the same primary defect, there is significant variable expressivity (Figure 1B). This implicates that despite the reduced genetic complexity shared by patients in same families, there are additional factors responsible for the adverse phenotype, beyond the very type of the mutation (see below). In recent years, several groups reported on smaller cohorts of patients with COL4A3/A4 heterozygous mutations that included subjects who progressed to more severe disease [12, 48, 63–65]. Particularly, in an Italian cohort of patients heterozygous for COL4A4 mutations, 9 of 37 (24.3%) progressed to ESKD. Similarly, the authors point out that their data make the differential diagnosis difficult with the benign familial hematuria due to heterozygous COL4A3/A4 mutations, especially in young patients, and with the XLAS in families where only females are affected [12].

**TBMN and genetic modifiers**

The expression of the full spectrum of symptoms in TBMN behaves as a complex multifactorial phenotype which depends on several genetic and environmental factors, most of which remain unknown. The glomerular obsolescence observed at later ages in a subset of TBMN patients and brought to closer attention in recent years, is one of these multifactorial traits which were at times attributed to the stochastic co-inheritance of another condition of glomerular origin while hypertension or FSGS due to obesity can also be contributory factors. The existence of genetic modifiers has been invoked by several investigators but it was only recently that reports presented experimental evidence for such modifiers.

Podocin is encoded by NPHS2 and is a component of the slit diaphragm of the GFB, interacting with nephrin, perhaps the most crucial component of the slit diaphragm. Mutations in NPHS2 are responsible for a recessive form of steroid-resistant nephrotic syndrome expressed in childhood, characterized by focal and segmental glomerulosclerosis. Variant p.R229Q, which decreases podocin binding to nephrin was shown to be associated with milder disease and later age at onset [66]. Initially, a report alluded to the potential role of variant p.R229Q in predisposing TBMN patients for proteinuria and renal failure [67]. Subsequently, we took advantage of a larger cohort of Greek-Cypriot TBMN patients that carried one of only three known mutations, including 77 patients that carried COL4A3-p.G1334E, and an additional familial hematuria cohort of 45 patients carrying a mutation in the CFHR5 gene. Investigation of this cohort showed that variant p.R229Q was significantly predisposing patients to a more adverse outcome with high grade proteinuria and ESKD at earlier ages [68]. With a minor allele frequency (MAF) of 2% this variant likely conforms to the rare variant-strong effect hypothesis. It is reasonable to hypothesize that this variant which on its own is of incomplete penetrance, is detrimental when co-inherited on the background of COL4 mutations that result in MH because of TBMN, on long follow-up. This same variant was incriminated in predisposing subjects of the general population to microalbuminuria and was associated with a 2.77-fold increased risk of presenting microalbuminuria even after adjustment for age, ethnicity, hypertension, obesity and diabetes in a multiple logistic regression model. Interestingly, subsequent work in a population of black and white middle-aged USA adults showed no significant association of p.R229Q with increased albumin-to-creatinine ratio or decreased eGFR [69, 70]. Additional studies on larger cohorts should be carried out in order to replicate this result and establish its role as a susceptibility factor in these conditions. Despite the fact that p.R229Q may turn out to be a good prognostic indicator for TBMN patients or patients with other glomerulopathies, it is certainly obvious that many more genetic modifiers exist as exemplified by the existence of severely affected patients who do not carry this variant and therefore more research is needed in well-defined cohorts. From the practical point of view, for patients who belong to families with familial MH because of TBMN that also segregate even low-grade proteinuria, caution should be exercised as well as closer follow-up. Perhaps, it is advisable to recommend at least one renal or a skin biopsy in one patient of familial hematuria, even before perceptible proteinuria ensues. The availability of genetic studies will hopefully distinguish those subjects who are heterozygous carriers of COL4 mutations, from those cases of X-linked or autosomal AS who admittedly run a higher risk for progressing to ESKD. The identification of genetic modifiers that act epistatically will enable us in the near future to prepare risk algorithms that will classify patients in high- and low-risk groups, of usefulness in genetic counseling and proper clinical attention.

In closing this section, it is worth pointing out the relatively high proportion of patients who develop CKD/ESKD because of TBMN (Figure 1). According to our data TBMN emerges as a much more frequent cause of CKD/ESKD compared with classical AS, either of X-linked or autosomal AS who admittedly run a higher risk for progressing to ESKD. The identification of genetic modifiers that act epistatically will enable us in the near future to prepare risk algorithms that will classify patients in high- and low-risk groups, of usefulness in genetic counseling and proper clinical attention.

**CFHR5 and C3 glomerulonephritis**

Complement is part of the innate immunity of primates and a very complex system with multiple players and multileveled regulation, serving as a first line of defense against pathogens. It is distinguished into three parts: the classical pathway, the mannose-binding lectin and the alternative pathway. They are all activated by distinct triggering events but all culminate in the formation of the membrane attack complex with C5b-9 as a major component for destroying the invading bacteria.

C3 glomerulonephritis (C3GN) and CFHR5 nephropathy are grouped under C3 glomerulopathy, which, according to a new classification, includes conditions with dysregulated alternative pathway of the complement, which in contrast to the classical system, does not require the involvement or the formation of immune complexes nor is it recognized by the
deposition of such antibody–antigen complexes. Grouped here is also dense-deposit disease with the characteristic dense osmiophilic intramembranous GBM deposits and isolated GBM C3 deposition with little or no immunoglobulin staining [71].

CFHR5 emerged as a key regulator of the alternative pathway, when some sequence variants were found to be associated with dense-deposit disease and atypical haemolytic uremic syndrome (aHUS) [72, 73]. The gene is a member of a family of CFH-related genes, CFHR1-CFHR5, mapping to chromosome 1q32 and sharing a number of sequence consensus repeats of 60 amino acids, ranging in number from 4 to 20, with CFHR5 comprising a combination of nine such repeats from the N-terminal, the middle and the C-terminal part of CFH [74]. The repeats contain recognition motifs for heparin and C3b of the mature convertase. CFHR5 protein has been co-localized with C3 in glomerular deposits of patients with glomerulonephritis [75].

This disease was reported in the past but nevertheless it was not clearly recognized that it can be transmitted as an autosomal-dominant nephropathy, presenting first with isolated MH since childhood. Synpharyngitic episodes of macroscopic hematuria following upper respiratory tract infections might have been misdiagnosed as IgAN, something that was also our experience until we realized the hereditary nature of the condition and the absence of positive immunofluorescence for IgA in kidney biopsies. The diagnostic dilemma was not completely settled until molecular testing revealed the inheritance of a CFHR5 mutation. In the absence of a diagnostic biopsy, clinically, the two conditions might appear as phenocopies of each other, although IgAN is not heritable.

In the recent classification, the disease is referred to as an inherited form of C3GN and was described as an endemic disease in Cyprus. It is really striking that nearly all patients described thus far are of Cypriot origin, living in Cyprus or London, which is known to host the largest number of Greek-Cypriots who emigrated there during two major waves, one during the middle of the last century and another one after the military invasion of Turkey to Cyprus, in July 1974. It was the group of British researchers who first identified patients in two families where renal biopsies demonstrated glomerular...
inflammation with complement C3 but not immunoglobulin or C1q deposited in the kidney [76]. There was a mild membranoproliferative glomerulonephritis (MPGN), also referred to as mesangiocapillary glomerulonephritis, with slight increase in mesangial cells and matrix. Some cells had slight capillary wall thickening. The EM showed subendothelial GBM electron-dense deposits, most likely corresponding to immunofluorescence C3 positivity and representing C3 cleavage products. It is worth mentioning that not all the biopsies in CFHR5 nephropathy actually show MPGN. DNA analysis identified a duplication of a large genomic region including exons 2–3 in the CFHR5 gene, encompassing the sequence consensus repeats 1–2. A larger protein was subsequently immunologically detected in the serum of affected subjects, along with the wild-type protein. No homozygous patient has been identified thus far despite a careful look. No ocular or hearing problems have been detected in patients who were examined nor have they self-reported such symptoms [76, 77].

During the past 20 years, we have been preparing a renal biobank in Cyprus, achieving genetic material and more recently additional biological samples for research purposes. It was stunningly interesting that when we searched for this exon 2–3 duplication in sporadic glomerulopathy samples, a few proved positive and further probing into their respective families demonstrated autosomal-dominant inheritance. Very quickly, we identified 21 families, all of Greek-Cypriot origin with a total of 130 live patients carrying this single mutation. A number of patients, who were tested, shared an extended haplotype of 8.74 cM, thereby indicating a common founder who probably lived around 11 generations ago. Apparently, the mutation originated in one of two villages in the Troodos Mountains of Cyprus, namely, Kalopanayiotis and Gerakies and subsequently it spread to another eight villages including areas around Nicosia the capital [78]. So far, no patients of another ethnic background have been identified with this exon 2–3 duplication in the CFHR5 gene or with any other mutation, and therefore, we cannot even estimate the world prevalence of this heritable condition. It is reasonable, however, to propose that similar forms of glomerular disease might be caused by other mutations of CFHR5 in unrelated individuals since patients with similar clinicopathological presentations have been reported in the past in several populations including Japanese and Caucasians [79, 80]. Currently, with 130 living patients in Cyprus among 659,350 Greek-Cypriots, according to the 2011 census, we estimate a prevalence of 1/5,072 individuals, rendering it one of the more common rare diseases.

The course of the disease is very similar to the one followed by TBMN, starting with MH in childhood and with no other symptoms until about 30 years, when proteinuria and CKD may ensue. Among all patients between 50 and 70 years of age, 1 of 3 developed CKD and 1 of 5 reached ESKD while 20% of patients across all ages reached ESKD (Figure 2A,B). In contrast to the corresponding TBMN group of patients, in CFHR5 nephropathy, the proportion of patients with proteinuria only, is very low in each age group, presumably because when there is progression of the disease, the patients develop CKD very soon after proteinuria ensues. Of particular importance is the gender difference in disease severity, as males are much more frequently affected with severe CKD and ESKD compared with females, for unknown reasons. Among all 18 patients who reached ESKD, only three were women. Kaplan–Meier analysis for renal survival shows that in men, by the age of nearly 80 years about half of patients progress to ESKD (Figure 2B).

Although no other unequivocal causative mutation has been described so far, a recent work reports on another CFHR5 variant in a heterozygous patient who presented with acute persistent glomerulonephritis following a streptococcal infection [81]. The patient had biochemical and histological findings consistent with C3GN, including reduced CFHR5 serum levels. However, the mother and half-sister of the index patient who also had inherited this variant did not have overt disease, while the same variant had been found previously in a sample that served as a control when they investigated the role of CFHR5 in aHUS. The authors speculate that even though this variant may not be sufficient on its own to precipitate the disease, it increases the predisposition and triggers the onset of CKD after streptococcal infection. There are additional reported rare defects which resulted in C3 glomerulopathies. A hybrid CFHR3–1 gene was found in one family with eight affected subjects and variable age of ESKD [82]. In another small family, the mother and her twin sons had inherited a C3 deletion, C3q22.2q1.4, and developed dense deposit disease due to alternative pathway dysregulation [83]. Finally, two female siblings were diagnosed in childhood with C3 deposition glomerulopathy because of inheriting a CFH deletion at position 224 [84].

In addition to the spectacular gender difference as regards progression to ESKD, the phenotypic heterogeneity is also a hallmark observation of CFHR5/C3GN. Taking advantage of the reduced genomic complexity as all patients carry an identical-by-descent mutation, we searched for genetic variants in genes of the GBF, as likely genetic modifiers. A cohort of CFHR5 patients, subdivided in mildly or severely affected, was added to a larger cohort with TBMN patients, and we showed that the p.R229Q podocin (NPHS2) variant predisposes to progressive disease, as evidenced by higher likelihood to develop proteinuria and CKD [68]. Also, in another approach we searched for miRNA-related variants and identified a SNP in the 3′UTR of the heparin-binding epidermal growth factor (HBEGF) gene, which is part of the miRNA hsa-miR-1207-5p seed region (miRNSP 1936 C/T, rs13385). HBEGF is expressed in podocytes and was shown to play a role in glomerular physiology [85]. Genetic association analysis of this variant in a cohort of 78 patients with CFHR5 nephropathy showed that the C allele protects from more severe progression of renal impairment on long follow-up. The same association could not be reached in a cohort of patients with mild/severe TBMN [86]. Functional studies using cultured undifferentiated podocytes, as well as luciferase constructs with sequences of the two alleles, corroborated the conclusion that the T allele interferes with the effective binding of the miRNA, thereby preventing the down-regulation of the HBEGF in cultured podocytes. This variant has a MAF of 16.6% in the Greek-Cypriot population and conforms more to the common variant-small effect
hypothesis [86]. We are not sure of the exact mechanism that this variant in HBEFG actually applies its modifying action. To our knowledge, this is the first report on the likely predisposing role of a miRSNP, acting on the background of a primary glomerulopathy.

CONCLUSIONS AND FUTURE DIRECTIONS

The familial hematurias of glomerular origin comprise a genetically and phenotypically heterogeneous group of conditions, where MH is invariably shared as a common early finding. The full spectrum of symptoms depends on multiple factors including the gene that is mutated, the position and type of the mutation, environmental factors and medication and admittedly variants in genetic modifiers that on their own cannot result in a Mendelian condition but can confer a high risk for an adverse outcome when co-inherited with another primary glomerulopathy. Five major genes are implicated in causing familial hematurias (FN1, COL4A3/A4/A5, CFHR5) and the list is bound to increase as there are more unmapped microhematuric families. Even though the finding of a causative mutation can establish unequivocally the diagnosis and some genotype–phenotype association has been attempted in COL4 mutations, this cannot as yet clearly predict the clinical course of the disease in individual patients, not even among patients in the same family who evidently share the same mutation. However, it is true that the presence of a family history of proteinuria and progressive CKD increases the risk, and it should alert the physicians and the family of the higher predisposition to more severe disease on long follow-up (for detailed guidelines for the management of AS and TBMN see FIGURE 2:

(A) Spectrum and distribution of symptoms according to age among 130 live patients with CFHR5/C3 glomerulonephritis, who are heterozygous carriers of a founder mutation, exon 2–3 duplication in CFHR5 (number of patients in parenthesis, on X-axis). Note the very similar course of disease as in thin basement membrane nephropathy, except that very few patients exhibit proteinuria without chronic renal failure, suggesting that once proteinuria ensues it triggers the impairment of renal function and leads to development of renal failure shortly thereafter. (B) Kaplan–Meier analysis of renal survival according to age in 136 CFHR5 nephropathy patients (68 males, lower line), men and women separately. Note the significant gender difference in renal survival, documented by a P < 0.001. In men, by the age of nearly 80 years about half of patients progress to end-stage kidney disease. Updated from Ref. [77].
Current nephrology has many laboratory experimental tools in investigating individual patients, including renal biopsy material and EM studies, immunohistochemistry studies and molecular genetics. Especially at early stages when MH appears as an isolated warning sign, it is worth having an algorithm dictating the rationale for deeper investigations. The DNA analysis, as the gold standard, is perhaps the only approach that can overcome the classical differential diagnosis in the absence of additional pathognomonic features and establish the diagnosis, among the most reasonable disorders. One advantage of utmost importance is that the establishment of the diagnosis by molecular testing in the first patient in a family may obviate the need for the invasive renal biopsy in the next relative who presents with MH and perhaps proteinuria. As emphasized in this review, however, the variable expressivity is so broad that the likely adverse outcome of severe CKD or even ESKD behaves as if it is a multifactorial trait. Notwithstanding that many patients belong to the same large family and share the same identical-by-descent mutation as well as a similar genomic background, there is manifestation of intra-familial phenotypic heterogeneity, alluding to the probable role of genetic modifiers (Figure 1B). Although there is evidence for two such putative modifiers, based on our own results, we anticipate the existence for many more, and we should keep our minds open for the existence of even common variants with strong effects waiting to be detected. In concluding this work, and with the realization that the genetic modifiers implicated so far need to be validated by independent research on separate patient cohorts, we suggest an algorithm which incorporates the molecular approach for a genetic diagnosis and for classifying the individual patient in a low- or high-risk group for progression (Figure 3). It should be

**Figure 3:** Algorithm for molecular testing of patients belonging to families segregating familial microscopic hematuria of glomerular origin. A kidney or a skin biopsy may be performed before or after the molecular analysis depending on the clinical status or disease progression of the patient, for histological evaluation. Appropriate immunohistochemical or immunofluorescence staining will differentiate between collagen IV nephropathies, CFHR5/C3 glomerulonephritis and glomerulopathy with fibronectin deposits. In familial cases with multiple affected subjects, a single early biopsy in the presence of isolated hematuria or low-grade proteinuria may guide molecular testing, in view of the genetic heterogeneity. In most, but certainly not all, families where a diagnosis has been established by molecular means, an invasive renal biopsy in additional patients may be rendered unnecessary. As genetic modifiers are being unraveled and validated, their inclusion in molecular testing may assist in better classification and treatment of patients. In case of extended founder mutations, initial testing should include those mutations before proceeding to more demanding investigative methodologies [45, 47, 50, 87, 88]. Updated from Ref. [50].
emphasized that we view genetic modifiers as hypomorphic DNA variants that in most cases exert an effect which is totally benign or not-perceptible on its own, however when co-inherited with another primary glomerulopathy it applies a degenerative detrimental role that takes many years to become manifest and result in a harmful clinical outcome, during the long aging process. The research community should be alerted to the fact that more genes exist that when mutated are responsible for familial forms of hematuria while polymorphic variants in each one of these could act as modifiers to one another. Finally, for patients with familial glomerular MH, with or without episodes of macroscopic hematuria the differential diagnosis includes (i) male patients with XLAS, (ii) heterozygous female carriers of an X-linked mutation, (iii) male and female patients with ARAS, (iv) male and female COL4A3/A4 heterozygous carriers, (v) since 2009 the newly described CFHR5 nephropathy (vi) and a rarer, well-defined glomerulopathy associated with MH, which is the GFND. A genetic diagnosis will promote the distinction between these conditions, will improve our prognosis for the individual patient and will promote early nephroprotective therapy.

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CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES


antibodies in diseases such as atypical HUS and potentially some of the complement mediated glomerulopathies (1).

Research has also identified a number of intriguing associations between the susceptibility and expression of CKD in African Americans and genetic mutations that may have had a protective role in the past against infectious disease such as the ApoL1 mutation and resistance to trypanosomiasis (2).

It seems as if the age of new genetics of kidney disease is dawning!

The NDTERA-EDTA OLA readers may be interested to learn more from the authors of this very interesting article about:

(1) Are the observed mutations of a number of components of the complement system linked to a protective role in our ancestors of these complement mutations in their interactions with infectious organisms? In other words, are the complement system mutations that are currently associated with a range of nephropathies the price we pay for the protection by these mutations of our ancestors against microorganisms?

Bacteria seem to mutate constantly to resist the lytic effect of complement system (3), one would expect counter mutations by the complement system to overcome these resistances?!

(2) Are there mutations that identify those patients with thin membrane disease who are likely to progress into CKD?

(3) Whether the time has come for more systematic genetic analysis of common and most likely polygenic kidney disease?

Rapid next generation genome sequencing (NGS) technology with systematic exome sequencing has come to age and may provide new insights into the role of genetic mutations in the susceptibility, initiation and progression of CKD (4).

Prof Meguid El Nahas

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