X-linked, *COL4A5* hypomorphic Alport mutations such as G624D and P628L may only exhibit thin basement membrane nephropathy with microhematuria and late onset kidney failure

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Abstract

Alport syndrome (ATS) results from X-linked, *COL4A5* mutations (85%) or from autosomal recessive homozygous or compound heterozygous *COL4A3/A4* mutations (15%), associated with alternate thinning and thickening as well as splitting and lamellation of the glomerular basement membranes. In contrast, familial microhematuria with thin basement membranes is thought to result from heterozygous *COL4A3/A4* mutations. This absolute separation may not always be true. Renal biopsies and molecular genetics were used to study microhematuric families in the Hellenic population we serve. The *COL4A5* gene was studied by PCR and direct re-sequencing for new mutations, while PCR-RFLP was used to identify more carriers of known *COL4A5* and *COL4A3/A4* mutations. Molecular genetics in two undiagnosed microhematuric Cypriot families, revealed *COL4A5* mutation P628L indicating X-linked ATS. Of nine males, seven developed end stage kidney disease (ESKD) between 31 and 56, while two are well at 51 and 57, exhibiting microhematuria and thin basement membrane nephropathy (TBMN). *COL4A5* mutation G624D was also identified in six Greek families. Seventy five members had DNA tests and 37 proved positive. Four positive males developed ESKD at 61, 51, 50 and 39 years, while the remaining and all females showed only microhematuria. A literature search revealed eight papers with six similar hypomorphic *COL4A5* mutations presenting as phenocopies of TBMN. In conclusion, X-linked *COL4A5* ATS mutations produce a phenotypic spectrum with a) classical ATS with early onset ESKD, neurosensory deafness and ocular defects b) males with only ESKD and late deafness and c) males due to missense mutations, such as G624D and P628L that may only exhibit microhematuria, TBMN, mild chronic renal failure (CRF) or late onset ESKD. Consequently when investigating “benign familial hematuria” these and other similar X-linked *COL4A5* mutations should also be searched for. Hippokratia 2013; 17 (3): 207-213

Keywords: Alport syndrome, benign familial hematuria, *COL4A3/COLA4/COL4A5* mutations, hypomorphic *COL4A5* mutations, phenotypic heterogeneity, thin basement membrane nephropathy.

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Introduction

Patients with X-linked Alport syndrome (ATS), due to *COL4A5* mutations on chromosome Xq22.3, usually present in childhood with continuous microscopic hematuria and episodes of macroscopic hematuria, often associated with pyrexia from upper respiratory tract infections (URTI) and other causes. This microscopic hematuria is usually followed in adolescence by proteinuria and hypertension that gradually progress to renal failure and end stage kidney disease (ESKD) by the 2nd or 3rd decades of life. High-tone neurosensory deafness develops in ~80% of such patients and ocular complications in ~50%. Classical perimacular dot-and-fleck retinopathy commonly develops in patients that develop early ESKD, before the age of 30 and usually results from serious, large deletions, frameshift and nonsense *COL4A5* mutations.

Steady progress in molecular genetics over the last 20 years, now enables confirmation of most X-linked ATS patients through identification of their underlying *COL4A5* mutations. In the past however, ATS diagnosis relied entirely on renal biopsy electron microscopy (EM) findings and a thickened, split and lamellated glomerular
basement membrane (GBM) was thought essential before the diagnosis of ATS could be made. Today, some 630 COL4A5 ATS mutations have been identified worldwide and a correlation has been attempted between the type and site of the underlying mutation and the resultant phenotype, particularly the age at which ESKD is reached. It is important to realize that COL4A5 mutations are all different and while most large deletions, frameshift and nonsense mutations are characterized by early ESKD, accompanied by ocular and neurosensory hearing deficits, a small number of X-linked ATS patients, due to milder, missense mutations, develop ESKD at a much later age or not at all. These hypomorphic mutations are not totally destructive but are found on genes encoding for proteins that maintain some residual function. They were used to be called “adult type” mutations and affected hemizygous males did not show ocular changes, while hearing loss developed late in life, or not at all. Molecular genetics have been vital in helping us understand the variable clinical phenotype of COL4A5 mutations which in some hemizygous males are characterized by mild GBM changes or even pure TBMN with only microscopic hematuria. This idea was unthinkable a few years ago, when microscopic hematuria and TBMN on EM, were believed to be caused only by autosomal heterozygous COL4A3/A4 mutations. A major change in our thinking and clinical approach is now warranted and Figure 1 attempts to describe and summarize this new concept whereby X-linked COL4A5 ATS mutations are responsible for a continuous phenotypic spectrum that starts with a) classical ATS, characterized by early ESKD, ocular defects and neurosensory deafness, b) moving to milder cases with variable to late onset kidney failure and tardive neurosensory deafness without ocular defects and c) ending through some mild, hypomorphic missense mutations, such as G624D, P628L and all other mutations mentioned in references 3,9,10,12, to TBMN and microscopic hematuria. These hypomorphic COL4A5 ATS mutations that encode for collagen IV molecules which retain some residual activity can lead to a phenotype in males that is identical to pure familial microhematuria, invariably produced by autosomal heterozygous COL4A3/A4 mutations. They actually present as phenocopies of TBMN.

In one of the earliest large series of 39 X-linked COL4A5 ATS mutations, published by Tryggvason’s group in 1998, two missense mutations, G624D and L1649R, stood out for failing to lead to early ESKD, ocular changes and hearing loss. Mutation L1649R, common in the western USA, had been identified even earlier, with the onset of renal failure in the 4th and 5th decades and an even later onset of neurosensory deafness. Chen et al in a Chinese paper in 2001, Wilson et al in 2007 in New Zealand and Kaneko et al in Japan in 2010, all described genetically proven X-linked ATS families that demonstrated benign familial hematuria and thin membrane nephropathy from three additional COL4A5 mutations, G156A, C1638Y and G1000V respectively. In 2007, Slajpah and colleagues re-discovered missense COL4A5 mutation G624D, in six Slovenian families. One family showed members with late onset renal failure but interestingly, the remaining five families behaved more like benign familial hematuria with TBMN and no documented as yet ESKD. Recently, we also demonstrated the presence of X-linked, ATS mutation G624D, in one Hellenic microhematuric family that exhibited a mild phenotype. Once this was appreciated, five more clinically unclear Hellenic microhematuric families were shown to carry this same mutation, raising the number to six and rendering this G624D hypomorphic mutation, the commonest ATS COL4A5 mutation in the whole of Greece. We also identified two new Cypriot microhematuric families due to a new “adult type” X-linked COL4A5 mutation, P628L, which in affected male Cypriots, leads to a benign and variable phenotype ranging from isolated, late onset ESKD to only microscopic hematuria and TBMN. In a recent report, we hypothesized that this milder phenotype associated with mutations G624D and P628L might be related to their position very close to the 12th natural interruption of the collagenous domain, thereby not interfering drastically with triple helix formation.

These new findings strengthen the new concept that some milder, hypomorphic X-linked COL4A5 mutations can lead to a phenotype, similar to that of autosomal heterozygous COL4A3/A4 mutations (Figure 1). It is important to also remember that while heterozygous COL4A3/A4 mutations in both males and females are universally associated with microscopic hematuria and TBMN, up to 30% of all such patients will develop

![Figure 1: This figure aims to transfer the new knowledge that different X-linked, COL4A5 ATS mutations may lead to: A) classical ATS with early ESKD, neurosensory deafness and ocular defects accompanied by widening, thickening, splitting and lamellation of the GBM. B) Hemizygous males with only late onset pure kidney failure without ocular defects and very rarely tardive neurosensory deafness with milder changes along their glomerular basement membranes. C) Lifelong microhematuria due to TBMN, occasionally accompanied with ESKD late in life or no ESKD at all. This phenotype results from some mild, missense mutations, such as G624D and P628L that maintain some residual protein activity. These hypomorphic COL4A5 mutations may also be common in patients with “benign familial hematuria.”](image-url)
ESKD by the age of 70 years, due to progressive focal segmental glomerulosclerosis (FSGS), occasionally with sensorineural deafness, most probably because of co-inherited nephrotoxic modifier genes. These patients and their families were described in the past as examples of autosomal dominant ATS.

Variable clinical phenotypes and mixed renal biopsy electron microscopy findings are now easier to understand, thanks to molecular genetics which has emerged as the most sensitive and accurate method for diagnosing collagen IV nephropathies. Molecular genetics testing accompanied by EM are clearly the best tools for understanding the different clinical phenotypes of ATS patients.

**Missense, mild COL4A5 mutation P628L in two Cypriot families**

Figure 2A describes Cypriot family CY4206 carrying X-linked COL4A5 mutation P628L. This mutation was identified in 2009 and it helped put an end to a long discussion over 19 years, regarding the precise aetiology and pathophysiology of this familial microhematuric nephropathy. Generation II includes eight siblings, six males, four molecularly checked and affected and two sisters, one being a proven heterozygous carrier. The 5th brother, who lives in the UK, also developed ESKD, presumably from the same cause and has been successfully transplanted though no DNA studies have been carried out so far. The 6th brother has not been investigated so far. Altogether, four male and four female family members were tested positive for mutation P628L. Brothers II-8 (11018) and II-10 (11019), both presented in 1990 with microscopic hematuria, proteinuria and renal insufficiency. Renal biopsies were carried out in March and Sept 1990, before the introduction of routine EM in our renal unit and the light microscopy findings showed non-specific changes of advanced renal failure. All five affected males reached ESKD at 30, 31, 34, 44 and 56 years of age. The somewhat delayed age at which ESKD was reached together with the absence of neurosensory deafness and ocular defects were interpreted over the years as evidence against the possibility that X-linked ATS was the underlying pathology. However the clear-cut molecular genetic results in 2009, proved beyond any doubt, the presence of X-linked ATS and strengthened the new concept that mild, hypomorphic COL4A5 mutation P628L with residual protein function may lead to a benign ATS phenotype without ocular and neurosensory deficits.

Figure 2B describes Cypriot family CY4212 that had been studied clinically for over a decade before the recently identified COL4A5 mutation P628L established the correct diagnosis of X-linked ATS. Generation II consists of eight siblings, six males, four being affected and two females, one exhibiting the mutation. Altogether nine mutation carriers, four males and five females have been identified so far and all exhibit microhematuria. The four ATS males also developed proteinuria and two patients,
UCY-1111 and UCY-1137 progressed to ESKD at ages 52 and 45 and died at 60 & 57 years of age respectively. The remaining two affected male patients are currently 51 and 57 year old and only show mild renal insufficiency, (creatinine: 1.6 & 1.5 mg/dl respectively). These two patients had renal biopsies that had shown well-preserved glomeruli with widespread thinning of the GBM on electron microscopy. The renal biopsies were carried out in 2002 and 2003 when no molecular genetics results were available and the precise diagnosis was not clear. Because of the presence of thin membranes in adulthood, X-linked COL4A5 ATS was thought unlikely at the time of the biopsies and the working diagnosis was TBMN due to a yet unknown heterozygous COL4A5/A4 mutation with slow progression to CRF \(^{14,16}\). One of these two patients later developed C-ANCA angiitis but responded well to treatment with pulses of IV cyclophosphamide and solu-medrol. No female carriers showed any progression to CRF and all maintain normal kidney function with only microscopic hematuria. A renal biopsy with EM in one such female patient showed TBMN. The correct molecular diagnosis of X-linked COL4A5 ATS finally settled a confusing clinical picture for over a decade. What is now new and educational is the new knowledge that TBMN with microhematuria and near normal kidney function or very mild CRF in adult life may result from a COL4A5, hypomorphic, mild missense mutation, such as P628L.

**Missense, mild, COL4A5 mutation G624D in six Greek families**

Our Unit acts as a referral center for inherited renal diseases in Greece and following our recent publications of familial microhematuria associated with heterozygous COL4A3/A4 mutations\(^{14,16}\) we have received DNA samples from several Greek families exhibiting microscopic hematuria and occasional late onset renal failure. One such family with an equivocal diagnosis was initially screened for linkage to either the autosomal COL4A3/A4 genes or the X-linked COL4A5 gene. Segregation with the autosomal genes was excluded. Subsequent examination of the COL4A5 gene revealed the G624D mutation. Following this finding\(^{13,17}\), we screened several more such microhematuric Hellenic families for the same G624D mutation and five more large families proved positive. The latest such family (GR4217) is illustrated in Figure 2C. Fifteen members were tested, six males and nine females with 10 of them being positive, two males and eight females, all showing microhematuria. Characteristically, one hemizygous male reached ESKD at 61 and four years later, he is currently alive on hemodialysis. The other affected male, age 32, only shows microhematuria. Altogether 75 family members, 28 males (12 positive) and 47 females (25 positive) from these six families were genetically tested. Of the 12 hemizygous males in these six Hellenic families, only four have reached ESKD at ages 61, 51, 50 and 39. These families are characterized by a fairly large size, female preponderance with only microhematuria, late onset ESKD in hemizygous males at a mean age of 50 years (39-61) and complete absence of ocular signs. Only two patients showed late onset neurosensory deafness.

**Other causes of familial microscopic hematuria (FMH) in Cyprus**

Familial microscopic hematuria (FMH) has been a major clinical and research interest in our renal unit in Cyprus since its inception in 1984 and Table 1 describes the currently four major causes. These are: a) heterozygous male and female carriers of COL4A3/A4 mutations b) mesangial C3 nephropathy due to a novel, exon 2-3 duplication mutation on the CFHR5 gene in the alternative complement pathway. c) Alport syndrome due to: i) X-linked COL4A5 mutations in hemizygous males and ii) autosomal recessive homozygous or compound heterozygous COL4A3/A4 mutations in both male and female patients and d) heterozygous female carriers of COL4A5 ATS mutations.

The identification of two lifelong microhematuric Cypriot sisters that reached ESKD in their late fifties in the mid-1980’s, was a major turning point in our studies, because gradual unraveling of their family over many years, led to a very large, five generation family with 67 studied members. Among 48 at-risk members, DNA studies revealed that 31 were positive carriers of mutation G1334E in COL4A3 and were characterized by early and lifelong microscopic hematuria, to which many years later proteinuria, hypertension, chronic kidney failure and ESKD from FSGS was added in a number of such patients (Figure 3). Several more similar Cypriot microhaematuric families with very late onset FSGS were gradually identified and by 2007, after several attempts to link these families to known candidate genes of familial FSGS had failed, these families with microhematuria leading to ESKD were successfully linked to heterozygous COL4A3/A4 mutations such as G1334E on COL4A3, G871C on COL4A3, 3533delC on COL4A3 and 3854delG on COL4A4\(^{14,16}\). Currently, 208 microhematuric patients in 22 Cypriot families are heterozygous for one of these four mutations. All carriers start with microhematuria in childhood and in all 22 families some carriers have subsequently developed proteinuria, hypertension and renal failure in their 40’s, 50’s and 60’s. Renal biopsies in 24 patients with proteinuria and renal insufficiency have all shown various stages of FSGS on top of TBMN observed in 22 biopsies where EM was available. There were no ocular ATS defects and late onset neurosensory deafness was present in only seven patients. Thus TBMN resulting from heterozygous COL4A3/A4 mutations has proven to be the commonest cause of FMH.

The second commonest cause of FMH appears to be the newly discovered familial C3/CFHR5 nephropathy that results from a novel, exon 2-3 duplication mutation on the CFHR5 gene in the alternative complement pathway. This autosomal dominant disease is present in 21 large Cypriot families with currently 136 living affected members. The histopathology is pure mesangial C3 neph-
Table 1: Familial microscopic hematuria is common in Cyprus. The four major causes are: 1) Thin basement membrane nephropathy (TBMN), due to heterozygous COL4A3/A4 mutations, 2) Mesangial C3, CFHR5 nephropathy, involving the complement alternative system 3) Alport syndrome due to: i) X-linked COL4A5 mutations in hemizygous males and ii) autosomal recessive homozygous or compound heterozygous COL4A3/A4 mutations in both male and female patients and 4) Heterozygous female carriers of COL4A5 ATS mutations.

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<th>Causes of Familial Microscopic Hematuria in Cyprus, 2012</th>
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<tr>
<td>1. TBMN</td>
<td>Four heterozygous COL4A3/A4 mutations</td>
<td>22 large families with 208 male &amp; female patients</td>
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<tr>
<td>2. Mesangial C3, CFHR5 Nephropathy</td>
<td>CFHR5 mutation affecting the complement alternative system</td>
<td>21 large families with 136 male &amp; female patients</td>
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<td>3. X-linked COL4A5 ATS &amp; Autosomal Recessive ATS</td>
<td>X-linked, COL4A5 P628L mutation Homozygous or compound heterozygous COL4A3/A4 mutations</td>
<td>2 families with 8 hemizygous males 2 families with 5 patients</td>
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<tr>
<td>4. X-linked ATS COL4A5 female carriers</td>
<td>X-linked, COL4A5, ATS female, heterozygous carriers</td>
<td>2 families with 9 female carriers</td>
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Table 2: Published X-linked, COL4A5 ATS mutations, characterized by absent or very late onset ESKD, absent ocular complications and absent or delayed neurosensory deafness. Ultrastructurally these patients show intermediate GBM changes and mostly present as phenocopies of TBMN with microscopic hematuria.

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<th>X-linked COL4A5 Mutations</th>
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| C1564S, L1649R, R1677Q | The three most common “benign” “adult type” X-linked COL4A5 mutations in Utah/USA. Delayed or absent ESKD with only late neurosensory deafness | Barker et al, 1996
d Pont-Kingdon et al, 2009
d Martin et al 1998 |
| G624D, P628L | Benign familial hematuria & diffuse thinning of the GBM. Benign clinical course with absence of or late ESKD Absent ocular complications and no neurosensory deafness | Martin et al, 1998
Slajpah et al, 2007
Demosthenous et al, 2012 |
| G156A | A Chinese family with TBMN and only microscopic hematuria | Chen et al, 2001 |
| C1638Y | A large family in New Zealand. Only 3 out of 8 males progressed to ESKD. No ocular problems or deafness | Wilson et al, 2007 |
| G1000V | A Japanese family with only benign familial hematuria | Kaneko et al, 2010 |

Nephropathy with similar C3 deposits along the GBM too, in the absence of any immunoglobulin deposits. The third cause of FMH appears to be the Alport syndrome, which appears rare on the island. Only X-linked, COL4A5 mutation, P628L has been detected so far in two families with eight affected hemizygous males. This hypomorphic COL4A5, P628L mutation is of great interest because of its benign phenotype, as two adult males show only microhematuria as a result of TBMN. Autosomal recessive ATS is also unusual with only 5 COL4A3/A4 homozygous or compound heterozygous patients recognised since 1984. The fourth cause of FMH refers to the female heterozygous carriers of X-linked COL4A5 ATS with nine such female patients identified in the two Cypriot families with the P628L mutation.

Discussion

Nephrologists and physicians in general, confronted with a family in whom some male and female members, exhibit microhematuria, without ocular complications or neurosensory deafness, may not immediately think of X-linked ATS, even if a male subsequently develops late onset renal insufficiency. According to classical teaching, the idea of X-linked ATS becomes even more remote, if the renal biopsy in such an adult with microhematuria ± mild renal insufficiency shows thinning of the basement membranes. The main aim of this review study is to help change this old rigid approach and make nephrologists worldwide realize that some COL4A5 missense and mild mutations are hypomorphic and consequently they may be responsible for TBMN with benign microscopic hematuria and occasional late onset kidney failure. The eight large families described in this paper, the six families described by Slajpah and the other families described by other investigators throughout the worldwide (Table 2) should make the nephrology community more aware of this entity, helping many such patients and their families reach the correct diagnosis early in order to receive proper medical care. Following a recent correlation analysis in 206 ATS patients from eminent Dr Flinter’s laboratory at Guy’s Hospital, London, UK, the recommendation was made that COL4A5 molecular studies should be requested if the patient meets at least two out of the four classical Alport diagnostic criteria. These four criteria are: a) positive family history of CRF and/or micro/macrophematuria, b) EM changes of ATS on renal biopsy, c) characteristic
There is, however, some phenotypic variation with some mutation carriers showing persistent pure microscopic hematuria up to the end of their lives, while some other mutation carriers develop additional proteinuria, hypertension, CRF and ESKD. COL4A3/A4 heterozygous mutations are the commonest cause of familial microscopic hematuria and TBMN in Cyprus. Such an approach is conservative and may be the explanation why some X-linked COL4A5 ATS patients with a mild phenotype are so slow to diagnose, most probably because they are not considered as likely X-linked ATS candidates and therefore not referred early for molecular COL4A5 genetic analysis. The end result is that such families may remain undiagnosed for long periods of time. The advent of next-generation sequencing (NGS) protocols enabling easier and fast detection of all possible variants in all three COL4A3/A4/A5 genes is indeed a promising development.

It is of interest that some X-linked COL4A5, ATS patients with a mild phenotype are so slow to diagnose, most probably because they are not considered as likely X-linked ATS candidates and therefore not referred early for molecular COL4A5 genetic analysis. The end result is that such families may remain undiagnosed for long periods of time. The advent of next-generation sequencing (NGS) protocols enabling easier and fast detection of all possible variants in all three COL4A3/A4/A5 genes is indeed a promising development that may help overcome this problem. It is of interest that unlike classical ATS with early onset ESKD, the patients in these families fulfill only one of the four classical ATS criteria and are characterized by late onset CRF or ESKD in their affected males. The mutations responsible for such milder phenotypes could be viewed as hypomorphic in the sense that the collagen IV molecules maintain some residual activity and the hemizygous males are not so severely affected as similar hemizygous males due to other more serious COL4A5, ATS mutations. As a result, the mildness of the phenotype may mislead the clinician who will diagnose TBMN rather than X-linked Alport. These scenarios delay family concerns and therefore allow for bigger families with geographical clustering.

The concept of hypomorphic mutations after the 1946 Nobel Prize winner Hermann J. Muller (1890-1967) has indeed helped us to understand the variable and milder phenotype of ATS and also that of other inherited nephrological diseases like polycystic kidney disease. Another probable explanation for the variable phenotypes of X-linked ATS might be the occurrence of somatic mosaicism for de novo COL4A5 mutations in sporadic ATS cases, when the mutation occurs as a somatic event post-conception and with unequal representation of the cells carrying the mutation in the various tissues and organs. An interesting case of a patient with such somatic and germline mosaicism was described for another collagen gene, COL1A1, in a patient with osteogenesis imperfect.

Persistent isolated microscopic hematuria in the first two decades of life is a urinary finding that unfortunately does not always attract the serious attention it should, including careful family studies for an underlying hereditary nephritis. Unlike proteinuria, microscopic hematuria does not often lead to an early renal biopsy which if and when attempted, should always include good EM studies. The main reason for not routinely carrying out renal biopsies in these young, pure microhematuric patients, is possibly the fact that pediatric nephrologists who usually see these patients first, do not often see the long term complications in these patients that usually begin in their late 30’s, 40’s and 50’s and therefore ascribe a benign nature to these microhematuric families. Indeed, as we and others have shown, several decades may elapse before additional pathological changes such as proteinuria, hypertension and renal insufficiency may develop. However, recent long term epidemiological follow up studies in Israel have confirmed that isolated microscopic hematuria in persons 16-25, was associated with a significantly increased risk of ESKD over a period of 22 years and hereditary nephritis was a major aetiological factor in these Jewish hematuric families in agreement with our studies in Cyprus.

Conflict of Interest
None to declare.

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