Genetics of anophthalmia and microphthalmia

Part 1: Non-syndromic anophthalmia/microphthalmia

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ABSTRACT

Eye formation is the result of coordinated induction and differentiation processes during embryogenesis. Disruption of any one of these events has the potential to cause ocular growth and structural defects, such as anophthalmia and microphthalmia (A/M). A/M can be isolated or occur with systemic anomalies, when they may form part of a recognizable syndrome. Their etiology includes genetic and environmental factors; several hundred genes involved in ocular development have been identified in humans or animal models. In humans, around 30 genes have been repeatedly implicated in A/M families, although many other genes have been described in single cases or families, and some genetic syndromes include eye anomalies occasionally as part of a wider phenotype. As a result of this broad genetic heterogeneity, with one or two notable exceptions, each gene explains only a small percentage of cases. Given the overlapping phenotypes, these genes can be most efficiently tested on panels or by whole exome/genome sequencing for the purposes of molecular diagnosis. However, despite whole exome/genome testing more than half of patients currently remain without a molecular diagnosis. The proportion of undiagnosed cases is even higher in those individuals with unilateral or milder phenotypes. Furthermore, even when a strong gene candidate is available for a patient, issues of incomplete penetrance and germinal mosaicism make diagnosis and genetic counselling challenging. In this review, we present the main genes implicated in non-syndromic human A/M phenotypes and, for practical purposes, classify them according to the most frequent or predominant phenotype each is associated with. Our intention is that this will allow clinicians to rank and prioritize their molecular analyses and interpretations according to the phenotypes of their patients.

Keywords: anophthalmia, microphthalmia, coloboma, eye development, genetics.

I - INTRODUCTION

Early eye development

The formation of the eye is complex, requiring coordinated interactions between a variety of tissues of different embryonic origins. These include the neuroepithelium, the surface ectoderm and the extraocular mesenchyme, the latter originating from the neural crest and the mesoderm (Fuhrmann 2010). Eye morphogenesis is highly conserved among vertebrates. It begins during gastrulation, when the eye-forming region (the eye field) is induced within the anterior part of the neural plate. In humans, this corresponds to the 4th week of gestation/embryonic development. During neurulation, the whole eye-forming region evaginates laterally, splitting the eye field into right and left optic vesicles. The optic vesicles interact with the overlying surface ectoderm, inducing its infolding, which ultimately gives rise to the lens and part of the cornea. Extension of the optic vesicle leads to the invagination of its distal surface into proximal and distal territories, forming the optic stalk and optic cup, respectively. The two layers of the optic cup form the neural and pigmented retina. This invagination is asymmetric and a furrow develops from the ventral side of the optic cup into the optic stalk, called the optic fissure. This transient opening enables the entry of the hyaloid artery to supply the developing eye. The inferior and most proximal invagination leads to the formation of the optic stalk, which allows the axons of the optic nerve to reach the brain. The nasal and temporal edges of the optic fissure subsequently fuse to close the layers of the eye during the 7th week of development in humans, establishing the basic structure of the eye (Mann 1953).

This process of eye formation is directed by a network of genes, and any disruption of these morphogenetic events by genetic or environmental influences can potentially cause growth and structural defects, such as anophthalmia, microphthalmia and coloboma.

Hypotheses for disruptive mechanisms

The pathophysiological mechanisms responsible for anophthalmia/microphthalmia (A/M) (absence or reduced growth of the ocular globe, respectively) remain poorly understood. Mann proposed that A/M may result from a lack of induction at the level of the primitive neural tube or a failure of the optic pit to enlarge and form the optic vesicle (Mann 1953). Later suggestions included secondary regression of an ocular structure during development (rather than primary optic vesicle aplasia), explaining the variable presence of buried ocular vestigial tissue in human anophthalmic sockets seen on histological examination (Fitzpatrick and van Heyningen 2005). Additional suggestions have included that anophthalmia can occur following failure of lens induction (Inoue et al. 2007), early retinal differentiation or disruption of the extensive cell movements that are integral to optic vesicle

invagination (Loosli et al. 2003; Stigloher et al. 2006; Winkler et al. 2000). Asymmetric involvement is common, suggesting differences in robustness and buffering mechanisms between the two sides.

Definitions

Anophthalmia and microphthalmia are the most severe developmental eye abnormalities and are frequently responsible for severe visual impairment, accounting for approximately 3% to 12% of visual impairment in children (Llorente-Gonzalez et al. 2011; Verma and Fitzpatrick 2007) and up to 20% when including coloboma (Shah et al. 2011b). They can be unilateral or bilateral, and when unilateral, the contralateral eye may be normal or have various ocular anomalies, including cataract or coloboma.

Anophthalmia corresponds to the total absence of any tissue of the eye or its associated structures. However, the term 'clinical anophthalmia' is used when visible ocular structures are absent, although a remnant may be histologically detectable. Therefore, there is a fine line dividing 'clinical anophthalmia' and 'extreme microphthalmia', although in the latter visible (rather than buried) ocular structures are present.

Microphthalmia refers to a decreased size of the eye. Although the diagnosis may be clear by observation, this can be clinically confirmed if the axial length of the eye is more than 2 standard deviations (SD) below the age-adjusted population mean; < 21 mm in adults and < 14 mm in newborns (Verma and Fitzpatrick 2007). Microphthalmia is classed as severe if the corneal diameter is < 4 mm and is associated with a total axial length < 10 mm at birth or < 12 mm after 1 year of age (Verma and Fitzpatrick 2007; Warburg 1993).

In addition, microphthalmia can be classified as simple or complex. Simple microphthalmia refers to an eye with reduced size, but which is anatomically intact. In contrast, when microphthalmia is associated with abnormalities of the anterior segment (Axenfeld-Rieger anomaly, Peters' anomaly, sclerocornea and cataract) or of the posterior segment (persistence of primitive vitreous, chorioretinal coloboma and retinal dysplasia), it is defined as complex. When microphthalmia is combined with an optic fissure closure defect, it is referred to as colobomatous microphthalmia. A coloboma is easily visible if it presents as a ventral gap in the iris, but it may also affect other more posterior structures of the eye, including the retina, choroid and optic nerve.

Nanophthalmia is a particular form of reduced eye size characterized by extreme hyperopia, microcornea and frequently glaucoma.

Posterior microphthalmia is an uncommon subtype of microphthalmia affecting only the posterior segment of the eye and is thus defined by a reduced total axial length in the presence of normal anterior segment dimensions, including corneal diameter, anterior chamber depth and anteroposterior length of the lens. This condition is frequently associated with high hyperopia and

abnormal retinal folds. The short distance between the lens and retina in eyes with posterior microphthalmia or nanophthalmia causes hyperopia with refractive errors ranging between +8.00 to +25.00 diopters.

Microphthalmia can also occur in association with primary congenital aphakia. This condition, resulting from a developmental arrest between the 4th and 5th weeks of gestation, is characterized by the absence of the lens. Aphakia can also be associated with other severe ocular abnormalities, such as sclerocornea or microcornea.

A/M can be detected prenatally using high-resolution ultrasound during the second or third trimester. Nevertheless, even experienced ultrasonographers can miss ocular anomalies, and routine anomaly scans often do not include detailed ocular evaluation. After birth, A/M is usually diagnosed by clinical ophthalmic examination. The detailed evaluation may include ultrasound to assess axial length and internal structure as well as electrodiagnostic testing to assess vision. The ophthalmologist will provide advice on early management of A/M and the socket(s), which may include referral to an appropriate specialist centre (Ragge et al. 2007). Systemic evaluation is important to delineate any associated conditions or identifiable syndrome (Slavotinek 2018). The visual consequences are highly dependent on the particular anomalies, therapeutic options available and whether the anomalies are unilateral or bilateral. For example, Shah *et al.* (Shah et al. 2011a) reported that 81% of microphthalmic eyes and 93% of microphthalmic eyes with coloboma had reduced vision.

Epidemiology

Several descriptive epidemiological studies have ascertained the prevalence of A/M. Most are based on national malformation registries and give a prevalence of around 1 to 3 per 10,000 live births (Bermejo and Martinez-Frias 1998; Busby et al. 1998; Chambers et al. 2018; Dolk et al. 1998; Kallen et al. 1996; Lowry et al. 2005; Morrison et al. 2002; Roos et al. 2016; Shah et al. 2011a; Spagnolo et al. 1994). Additional anomalies of other organ systems are present in 32-93% of A/M cases (Chambers et al. 2018; Roos et al. 2016; Shah et al. 2011a; Slavotinek 2011; Spagnolo et al. 1994; Tucker et al. 1996) and 20% of children with A/M and/or a coloboma have delayed psychomotor milestones (Morrison et al. 2002).

Etiologies

The etiology of A/M can include environmental and/or genetic factors. Environmental factors are believed to represent only a small proportion of the causes of A/M (Bermejo and Martinez-Frias 1998), the best known examples being certain infectious agents (e.g. rubella, CMV and toxoplasmosis) (Duszak 2009; Kava and Nagarajan 2009; Suhardjo et al. 2003), toxic substances (e.g. alcohol), and drugs (e.g. retinoids and thalidomide) with most of the epidemiological studies relating to an era

before the identification of genes for A/M (Lammer et al. 1985; Stromland 2004; Stromland et al. 1991; Stromland and Miller 1993). Although little is known about an association between smoking and A/M, maternal smoking in early pregnancy appears to be associated with an increase in the risk of A/M in the absence of a coloboma (Kallen and Tornqvist 2005).

Genetic alterations are now known to be a major cause of developmental eye anomalies. A/M can be isolated or associated with extraocular anomalies, when they may form part of a recognizable syndrome (Slavotinek et al. 2018). In recent work, Chambers (2018) reported 1,262 live births with A/M in Texas from a population of 4,207,898 during a ten year period. Approximately half (N = 608, 48.2%) were syndromic (i.e. occurred with a chromosome abnormality, malformation syndrome, or complex). The most common chromosome abnormality was trisomy 13 (N = 124, 20%).

Many chromosomal abnormalities have been associated with A/M. These may be visible on a standard karyotype or identified by array comparative genomic hybridization (aCGH), SNP array or whole genome sequencing (WGS) techniques. The rate of detection of chromosomal anomalies in patients with syndromic ocular involvement is 7-15% using conventional cytogenetics (Kallen and Tornqvist 2005; Roos et al. 2016). These include aneuploidy (mainly trisomy 13 and 18), triploidy and certain microdeletion or microduplication syndromes, such as 4p- syndrome (MIM#194190) or duplication of 3q, 4p or 10q regions. The introduction of aCGH has made it possible to identify cryptic chromosomal abnormalities in 10-15% of patients with syndromic ocular involvement and normal karyotyping (Balikova et al. 2011; Delahaye et al. 2012). However, in non-syndromic A/M the frequency of chromosomal abnormalities identified by aCGH is low (Raca et al. 2011). In a registry study of children born with anophthalmia, microphthalmia and coloboma, Roos *et al.* (Roos et al. 2016) reported chromosome microarray analysis had been performed on around 9% with a possibly pathogenic copy number variant observed in almost half of cases. Another study performed SNP array screening of 60 individuals with isolated (n=25) or syndromic A/M (n=35) and identified four causative and six potentially causative copy number variants in ten cases (17%) (Schilter et al. 2013). In addition to chromosomal abnormalities, structural and sequence alterations of a highly heterogeneous collection of single genes have been found to play a role in A/M, and interactions between these genes are beginning to reveal networks controlling eye morphogenesis. Overall, genetic variants seem to be the predominant cause of both syndromic and non-syndromic A/M.

The main causative genes encode **i)** transcription factors: *SOX2* (MIM*184429), *OTX2* (MIM*600037), *PAX6* (MIM*607108), *RAX* (MIM*601881), *VSX2* (MIM*142993), *FOXE3* (MIM*601094), *VAX1* (MIM*604294), *ATOH7* (MIM*609875), *SALL2* (MIM*602219), *SALL4* (MIM*607343), *MAF* (MIM*177075), *HMGB3* (MIM*300193), *SIX3* (MIM*603714), *SIX6* (MIM*606326), *PAX2* (MIM*167409), *PAX3* (MIM*606597), *MITF* (MIM* 156845), *TFAP2A* (MIM* 107580), *SOX10* (MIM*602229) **ii)** expression regulators: *YAP1* (MIM*606608), *BCOR* (MIM*300485), *CHD7* (MIM*608892) or **iii)** proteins involved in signaling pathways: *BMP4* (MIM*112262), *BMP7*

(MIM*112267), *SHH* (MIM*600725), *PTCH1* (MIM*601309), *GDF3* (MIM*606522), *GDF6* (MIM*601147), *MFRP* (MIM*606227), *LRP2* (MIM*600073). Recently, mutations in genes involved in the metabolism of retinoic acid, *STRA6* (MIM*610745), *ALDH1A3* (MIM*600463), *RARB* (MIM*180220) and *RBP4* (MIM*180250), have also been identified in patients with A/M. Nevertheless, many genes are involved which do not fall into these categories, for example: *PORCN* (MIM*300651), *COL4A1* (MIM*120130), *NAA10* (MIM*300013), *FRAS1* (MIM*607830), *FREM1* (MIM*608944), *PXDN* (MIM*605158), *PRSS56* (MIM*613858), *ABCB6* (MIM*605452), *ACTG1* (MIM*102560), *ACTB* (MIM*102630), *MAB21L2* (MIM*604357), *SMOC1* (MIM*608488), *HCCS* (MIM*300056), *COX7B* (MIM*300885), *C12ORF57* (MIM*615140), *TMX3* (MIM*616102), *FNBP4* (MIM*615265), *TENM3* (MIM*610083) and *TMEM98* (MIM*615949), *RAB3GAP2* (MIM*609275), *RAB3GAP1* (MIM*602536), *RAB18* (MIM*602207), *TBC1D20* (MIM*611663), *SMCHD1* (MIM*614982), *OLFM2* (MIM*617492).

II - DIAGNOSTIC STRATEGY

Recent advances in DNA sequencing technologies have significantly increased our knowledge of the genes underlying A/M (Plaisancie et al. 2016b). The importance of an accurate genetic diagnosis cannot be underestimated in terms of management, screening for associated conditions, genetic counselling and prenatal diagnosis. Although hundreds of genes have been suggested as being involved in eye development, around 30 of them have been repeatedly implicated in non-syndromic A/M families. In addition, some syndromic eye malformation genes have variants associated with only an eye phenotype (Slavotinek 2018). Overall, mutations in each gene explain only a small percentage of cases. According to the 2015 revision of GeneReviews (https://www.ncbi.nlm.nih.gov/books/NBK1116/), the major gene responsible for A/M is *SOX2*, accounting for 10-15% of affected individuals, usually those with a severe A/M phenotype. The next most frequent genetic causes are alterations in *OTX2* (2-5% of cases), *RAX* (3% of cases), *FOXE3* (2.5% of cases) and *PAX6* (2% of cases). Overall, the genetic cause is currently only determined in around 20-30% A/M patients, although this figure is higher in cases of severe and/or bilateral A/M (Chassaing et al. 2014; Gerth-Kahlert et al. 2013). For instance, in our series of 150 A/M patients of mixed severity (Chassaing et al. 2014) screened for mutations in *SOX2, OTX2, RAX, FOXE3, PAX6, GDF6* and *VSX2* by direct sequencing and semi-quantitative multiplex PCR, causative mutations were detected in 21% of patients. The proportion of individuals for which mutations were detected was higher in anophthalmic patients (54%) compared to those with unilateral microphthalmia (10%). Since this publication, mutations in other genes have been identified in an additional 10% of our patients (unpublished data), increasing the detection rate to about 30%. In another series including 51 probands with A/M (Gerth-Kahlert et al. 2013), aCGH and screening of a limited number of genes (*SOX2, OTX2, RAX, FOXE3, PAX6, BMP4, SMOC1* and *STRA6*) resulted in a diagnostic rate of 75% in patients with bilateral and severe forms of A/M and 20% in patients with unilateral and less severe forms. Gene screening alone allowed the identification of the genetic cause in around 30% of patients with severe phenotypes and around 10% for unilateral or less severe ones. In addition to the major genes listed above, other genes are also believed to explain a significant

proportion of cases. For example, based on their series of 75 A/M index cases and including a case they had previously reported (Yahyavi et al. 2013), Abouzeid *et al.* (Abouzeid et al. 2014) suggested that mutations in *ALDH1A3* are a frequent cause of A/M and responsible for approximately 10% of cases in consanguineous pedigrees. Of note, mutations in *ALDH1A3* have mainly been identified in consanguineous pedigrees.

Only a few studies have determined the mutation detection rate using whole exome sequencing (WES) in ocular developmental disorders. Slavotinek *et al.* (Slavotinek et al. 2015) screened 28 patients with A/M using WES, leading to a reliable molecular diagnosis in 4 individuals (14%). Deml *et al.* (Deml et al. 2016) performed WES for 28 A/M probands known not to carry mutations in *SOX2* or *FOXE3*. This data was analyzed prioritizing 83 known A/M genes and resulted in the identification of causative mutations in 3 individuals (11%). More recently, WES was applied to a cohort of 14 patients with bilateral A/M (Matias-Perez et al. 2018). As expected, given the bilaterality and severity of ocular phenotype, the mutation detection rate in this cohort was almost 60% (8/14), the majority carrying alterations in well-known eye genes (7/8). Thus, WES studies resulted in a detection rate similar to that obtained by the targeted sequencing of a panel of the main known genes. However, an advantage of WES over panel sequencing is the opportunity to identify new causal genes in particular pedigrees (Chassaing et al. 2016b; Matias-Perez et al. 2018; Patel et al. 2018; Srour et al. 2013).

Even if, theoretically, WES approaches were considered the best strategy to identify causative mutations (since 90% of pathogenic mutations are estimated to be located in coding sequences), there is an increasing recognition of the involvement of gene regulatory regions in pathological processes. Indeed, many studies have shown that variants in non-coding regions of the genome can cause ocular disorders (Bhatia et al. 2013; Chatterjee and Pal 2009; Cipriani et al. 2017; Conte et al. 2015; Davidson et al. 2016; Small et al. 2016; Volkmann et al. 2011). Although ocular genetic diseases generally follow a Mendelian pattern of inheritance, mutations in the non-coding regions of the genome (as well as some coding regions) have also been associated with non-Mendelian inheritance (Medina-Trillo et al. 2016). Variants in non-coding regions (~98% of the genome) represent almost 2% of known gene disruptions responsible for human inherited disease according to the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php). However, it is likely that their involvement is underestimated, since regulatory mutations are not currently prioritized in genetic analyses. Increasing the use of WGS in patients will allow for a more comprehensive evaluation of the proportion of variants in regulatory sequences that cause human pathologies.

Although no studies investigating A/M using WGS have been published to date, we hypothesize that they would demonstrate a much higher mutation detection rate than WES approaches due to their ability to analyze the regulatory regions of known A/M genes. WGS would also avoid biases specific to WES techniques, such as exon capture failings.

Therefore, WGS technologies provide increased opportunities for variant identification in both coding and non-coding regions of known and new A/M genes. However, the question of how these variants are interpreted and classified is of fundamental importance in providing reliable molecular diagnoses and appropriate genetic counselling. While genetic variants, whether sequence changes or CNVs, are generally easy to detect, the understanding of functional consequences and determination of causality is far more laborious. Furthermore, interpreting the deleterious effects of mutations in non-coding regions (deep intronic, promoter and *cis*-regulatory sequences) remains challenging.

The thorough interpretation of the impact of genetic variants has two key aspects. First is to predict the molecular impact of a variant. Guidelines for such predictions have been provided by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (Richards et al. 2015). These establish five classes of variants: benign, likely benign, of uncertain significance, likely pathogenic and pathogenic. In cases of variants of uncertain significance, additional *in vitro* or *in vivo* analyses can be undertaken to further establish their pathogenicity.

Second, but no less important, is the clinical knowledge of the phenotypes associated with specific A/M genes and mutations, and therefore the potential genotype-phenotype correlations. The causality of a pathogenic variant is relatively straightforward to assign when the phenotype of the case is distinctive and matches that of other individuals with mutations in the same gene, and where the variant segregates according to the expected inheritance pattern for the disorder. However, a molecular diagnosis is harder to provide when **i)** a variant is of uncertain pathogenicity, **ii)** a second mutant allele is not found in cases of recessively inherited phenotypes, **iii)** the genotype is unexpected given the phenotype, **iv)** no segregation pattern can be established e.g. in singleton cases or **v)** the variant (if heterozygous in the case of a dominant phenotype) is inherited from an unaffected parent. In such situations, a precise phenotypic description will assist in interpreting the molecular data. Indeed, where specific phenotypes are associated with particular genes, such precise descriptions can guide further genetic analyses, such as by extending analyses to include RNA studies in the case of splice site variants, or functional studies for regulatory region variants.

III - GENES IMPLICATED IN DISTINCTIVE OCULAR PHENOTYPES (Table 1)

Anophthalmia and severe microphthalmia

Anophthalmia and severe microphthalmia are at the most severe end of the A/M and coloboma spectrum and the genes responsible for the majority of these phenotypes are presented in the following section. Therefore, screening of these genes should be prioritized in patients with these phenotypes, especially as they also represent the genes most frequently involved in A/M and coloboma in general.

***SOX2* (*SRY [sex-determining region Y]-box 2*)**

SOX2 codes for a transcription factor which is highly expressed during ocular development (Fantes et al. 2003; Hever et al. 2006; Ragge et al. 2005b; Williamson et al. 2006). Its involvement in A/M was initially demonstrated in a patient with a ~740 kb deletion on chromosome 3 that included *SOX2* (Fantes et al. 2003). Subsequently, heterozygous point variants in this gene were identified in additional patients (Fantes et al. 2003; Ragge et al. 2005b). It has since been found that heterozygous *SOX2* pathogenic variants, including whole gene deletions, are present in 10-15% of all A/M patients (Bakrania et al. 2007; Gerth-Kahlert et al. 2013) making it the most common genetic cause for these disorders.

More than 100 different truncating and missense variants of this single-exon gene have been described [LOVD-SOX2]. Deletions of *SOX2* are also frequent and account for 25-50% of pathogenic variants identified in this gene (Bakrania et al. 2007; Chassaing et al. 2014). It is also of interest that most of the intragenic variants are private, having only been identified in individual families (Ragge et al. 2005b). The initial phenotypes associated with *SOX2* mutations included ocular anomalies (typically severe and bilateral), psychomotor delay, seizures, periventricular heterotopias, hippocampal and pituitary anomalies, growth retardation, genital and renal abnormalities, and some minor craniofacial findings (Ragge et al. 2005b). It was later shown that *SOX2* variants can also cause a syndromic form of A/M, Anophthalmia-Esophageal-Genital (AEG) syndrome (Williamson et al. 2006) (MIM#206900), and the same variant can be responsible for AEG syndrome or other *SOX2* phenotypes (Bakrania et al. 2007). Various other anomalies have also rarely been associated with these variants (Chassaing et al. 2007; Kelberman et al. 2006).

The spectrum of ocular anomalies associated with *SOX2* variants has progressively widened, with patients also displaying less severe phenotypes, including iris and retinal coloboma and iris hypoplasia (Chassaing et al. 2007; Wang et al. 2008), and in some cases no ocular anomalies. For example, one patient with a truncating mutation showed no overt or microscopic eye involvement (Chassaing et al. 2007). Also, one individual with a nonsense variant had non-syndromic hypogonadotropic hypogonadism and a seizure disorder, but no ocular abnormalities except for decreased thickness of the retinal nerve fiber layer (Shima et al. 2017). A further three individuals from a cohort with intellectual disability/developmental delay, but without anophthalmia or microphthalmia, were also shown to harbour point mutations or microdeletions of *SOX2* (Dennert et al. 2017) and in one case with a superior chorioretinal defect involving the optic disc and macula associated with a retinal dystrophy on normal sized ocular globes (Bakrania et al. 2007). Individuals with *SOX2* mutations may show later neurodegeneration, as indicated by a single case report (Ragge et al. 2013).

In summary, the ocular features associated with *SOX2* mutations are classically bilateral and severe (anophthalmia in the majority of patients). The most common extraocular features are psychomotor delay (mild to severe impairment), cerebral involvement (ventriculomegaly, hypoplasia of the corpus

callosum), hypogonadism and growth retardation, possibly related to pituitary anomalies, and renal anomalies. Rarely patients may also display esophageal atresia (Chassaing et al. 2014), sometimes as part of the AEG syndrome. A genotype-phenotype correlation has been suggested (Schneider et al. 2009), however patients with similar mutations have variable ocular and extraocular involvement and intra-familial clinical variability has also been frequently noted (Chassaing et al. 2007; Kelberman et al. 2006; Mihelec et al. 2009; Zenteno et al. 2006). In addition, the ocular features can be significantly asymmetrical between both eyes of the same patient, making attempts at genotype-phenotype correlations difficult. *SOX2*-related anophthalmia syndrome is transmitted in an autosomal dominant pattern, with the vast majority of patients having *de novo* variants. However, reported cases of germinal mosaicism (Chassaing et al. 2007; Faivre et al. 2006; Schneider et al. 2009) should be taken into account when providing genetic counselling.

***OTX2* (*orthodenticle homeobox 2*)**

OTX2 encodes a transcription factor involved in the formation of multiple ocular structures and is particularly expressed during the differentiation of the retina (Hever et al. 2006). The involvement of *OTX2* in A/M was demonstrated by Ragge *et al.* (Ragge et al. 2005a) using a positional candidate gene approach leading to the identification of variants in this gene in 8 A/M families. *OTX2* is now estimated to underlie 0.7-10% of A/M (Gerth-Kahlert et al. 2013; Henderson et al. 2009; Ragge et al. 2005a; Schilter et al. 2011; Wyatt et al. 2008). More than 70 different genetic alterations have been described (Human Gene Mutation Database [HGMD®], Stenson et al. 2017), including pathogenic truncating and missense variants, and gene deletions (Chassaing et al. 2014; Chassaing et al. 2012; Gerth-Kahlert et al. 2013), the latter representing 30-40% of variants in this gene (Chassaing et al. 2014; Wyatt and Ragge 2009).

The ocular phenotype of patients with pathogenic *OTX2* variants is highly variable even within the same family. It is often severe and ranges from bilateral anophthalmia (Fig. 1a) or Leber congenital amaurosis through to no ocular malformation (Chassaing et al. 2014; Ragge et al. 2005a), making genetic counseling very challenging. Other anomalies may include colobomas (iris and/or retinal), cataract and sclerocornea. The presence of extraocular anomalies is inconsistent, but can include intellectual deficiency, growth retardation and pituitary involvement, and deafness (Ragge et al. 2005a; Schilter et al. 2011; Wyatt et al. 2008). Mutations of *OTX2* have also been described in some patients with isolated hypopituitarism (Tajima et al. 2013). Furthermore, pathogenic variants in this gene have been identified in agnathia, a major malformation of the mandible during embryonic development (Chassaing et al. 2012; Patat et al. 2013).

The pattern of inheritance related to phenotypes caused by *OTX2* mutations is autosomal dominant. However, penetrance is incomplete and clinical variability common (Somashekar et al. 2017).

Mutations can be inherited or occur *de novo* (approximately 50% of cases). Cases of germinal mosaicism are also well described (Ragge et al. 2005a; Wyatt et al. 2008).

***RAX* (*retina and anterior neural fold homeobox*)**

RAX encodes a transcription factor whose role in ocular development has been studied in a variety of vertebrate models (Bailey et al. 2004). The gene is expressed early in the eye primordia and plays animportant role in the establishment and/or proliferation of retinal progenitor cells (Mathers et al. 1997).

Biallelic mutations have been identified in 7 families with a bilateral and severe ocular A/M phenotype (Abouzeid et al. 2012; Chassaing et al. 2014; Lequeux et al. 2008; Voronina et al. 2004) and in 1 family with isolated unilateral coloboma and retinoschisis (Huang et al. 2017). These mutations are of varying nature (missense, nonsense, frameshift and splicing mutations, and a gene deletion), but all act through a "loss-of-function" mechanism. In our experience, biallelic *RAX* variants are identified in approximately 3% of A/M patients (4/150) (Chassaing et al. 2014). Associated extra-ocular phenotypes are also varied. Intellectual disability was described in 3 patients from the 8 families described above. In comparison, in a separate study, no extra-ocular phenotype was reported in two patients, apart from frontal and sphenoid sinus abnormalities (Abouzeid et al. 2012).

Heterozygous mutations of *RAX* have been described in patients with chorioretinal coloboma (London et al. 2009) and A/M (Gonzalez-Rodriguez et al. 2010). However, for these patients the segregation pattern is unknown, and the link between these mutations and the ocular phenotype remains unclear. Heterozygous carriers of *RAX* mutations in A/M families with biallelic affected individuals lack any ocular phenotype. While it is difficult to exclude the possibility of dominant mutations, current evidence indicates that genetic counseling should tend towards considering *RAX* mutations as acting through an autosomal recessive mode of inheritance.

***VSX2* (*visual system homeobox 2*)**

VSX2, also known as *CHX10*, encodes a transcription factor that plays a major role in the development of mammalian eyes (Liu et al. 1994). *VSX2* is expressed during optic vesicle formation, and its expression in retinal progenitors allows their proliferation and differentiation into neuroretinal cells (Liu et al. 1994). The first mutations of *VSX2* were described in microphthalmic patients by Ferda Percin *et al.* (Ferda Percin et al. 2000). Since then, *VSX2* mutations (both missense and truncating) have been described in several other families with eye anomalies (Ammar et al. 2017; Burkitt Wright et al. 2010; Chassaing et al. 2014; Iseri et al. 2010; Khan et al. 2013; Reis et al. 2011a; Ullah et al. 2016). The

phenotype associated with mutations in *VSX2* is typically bilateral A/M, frequently associated with coloboma and sometimes with cataract, glaucoma and retinal dystrophy. Atypical ocular involvement has been described in one patient: his phenotype included subluxation of the lens, retinal dystrophy and severe myopia (Khan et al. 2013). Associations with delayed development and autistic spectrum disorder have occasionally been reported. However, extraocular involvement is uncommon. The A/M phenotype attributable to *VSX2* mutations is typically recessively inherited. However, interestingly one family had affected carrier parents with a semidominant inner retinal dystrophy, more severely affected offspring with severe microphthalmia and coloboma and a homozygous *VSX2* frameshift mutation (c.249delG p.[Leu84SerfsTer57], NM_182894) (Iseri et al. 2010).

***STRA6* (*stimulated by retinoic acid 6*)**

Four genes in the retinoic acid synthesis pathway are known to be involved in the A/M phenotype: *STRA6, RARB, ALDH1A3* and *RBP4*. Both loss and gain of retinoic acid signaling cause structural defects of organs, such as the eye, heart, lung, diaphragm and limbs, indicating that this pathway is vital for the development of these structures (Cunningham and Duester 2015).

STRA6 codes for a transmembrane receptor that mediates the cellular uptake of Vitamin A (retinol). Biallelic mutations in *STRA6* were first described in Matthew-Wood syndrome (Chassaing et al. 2009; Pasutto et al. 2007; Seller et al. 1996), later referred to as PDAC to reflect its major phenotypic components: (Pulmonary agenesis or hypoplasia, Diaphragmatic hernia, Anophthalmia/ microphthalmia and Cardiac defects) (Chitayat et al. 2007). However, in 2011 Casey *et al.* (Casey et al. 2011) demonstrated the contribution of biallelic *STRA6* mutations to isolated forms of microphthalmia, a finding later confirmed by Slavotinek *et al.* (Slavotinek et al. 2015). Ocular involvement is a constant feature of the phenotype associated with biallelic *STRA6* mutations. This can manifest as unilateral microphthalmia or anophthalmia, sometimes with contralateral eye defects, such as coloboma (Casey et al. 2011). Nevertheless, when present, microphthalmia is typically severe and bilateral. Interestingly, the phenotypic spectrum has been further broadened with the report of 3 patients with contractures and camptodactyly, in addition to PDAC (Marcadier et al. 2016).

STRA6-related eye disorders are inherited in an autosomal recessive manner. Nevertheless, ocular anomalies have been described in heterozygous carriers of some families. These include one with congenital cataract (Chassaing et al. 2013), one with bilateral coloboma of the iris and the retina (Golzio et al. 2007) and one with a right optic disc coloboma and left microphthalmia with iris coloboma (Ng et al. 2013). In addition, a heterozygous *STRA6* missense mutation was identified in a patient with bilateral microphthalmia and coloboma, unilateral retinal detachment, right-sided aortic arch, vascular ring and intellectual disability. This mutation was inherited from their mother, who also had bilateral microphthalmia and did not carry a second mutation (Slavotinek et al. 2015).

***RARB* (*retinoic acid receptor beta*)**

RARB is a thyroid-steroid hormone receptor and part of a superfamily of nuclear transcriptional regulators. In 2013, Srour *et al*. (Srour et al. 2013) reported a non-consanguineous family containing 4 siblings with bilateral microphthalmia and variable additional features of the PDAC spectrum (Chitayat et al. 2007). One of the affected individuals was known to lack mutations in *STRA6*. WES identified biallelic truncating mutations in *RARB* that segregated with the disease (c.355C>T p.[Arg119Ter] and c.1201_1202insCT p.[Ile403SerfsTer15], NM_000965). Sanger sequencing of *RARB* in an additional cohort (15 subjects) with A/M and at least one other feature of the PDAC syndrome identified *de novo* missense mutations (c.1159C>T p.[Arg387Cys] and c.1159C>A p.[Arg387Ser], NM_000965) in 3 additional sporadic cases (Srour et al. 2013). Targeted and exome sequencing have since resulted in the identification of one of these missense variants (p.[Arg387Cys]) and a further two *de novo* missense mutations (c.887G>C p.[Gly296Ala] and c.638T>C p.[Leu213Pro], NM_000965) in a total of 10 additional individuals (Slavotinek et al. 2015; Srour et al. 2016). Thus, to date there is only one family with a PDAC phenotype related to biallelic *RARB* mutations. Therefore, Srour *et al.* (Srour et al. 2016; Srour et al. 2013) suggest that *RARB* could underlie both recessive and dominant forms of the PDAC phenotype, with the *de novo* missense variants in *RARB* acting through a "gain-of-function" mechanism. Concerning the *de novo* cases, the microphthalmia phenotype was bilateral in 11/13 subjects. In addition, most subjects had sclerocornea (10/11) and coloboma (7/10) (Srour et al. 2016). However, in contrast to *STRA6* (Casey et al. 2011), there is currently no report of an isolated A/M phenotype associated with mutations in *RARB*. For example, Srour *et al.* (Srour et al. 2013) sequenced *RARB* in 11 cases with isolated bilateral A/M, but did not find any mutation. Moreover, in addition to the PDAC spectrum of anomalies, all the subjects described by Srour *et al*. (Srour et al. 2016) who survived beyond the neonatal period also manifested severe global developmental delay combined with a progressive motor impairment and associated spasticity and/or dystonia (with or without chorea).

***ALDH1A3* (*aldehyde dehydrogenase 1 family member A3*)**

ALDH1A3 encodes a retinaldehyde dehydrogenase, an enzyme involved in the metabolism of Vitamin A to retinoic acid (Cunningham and Duester 2015; Cvekl and Wang 2009). Fares-Taie *et al.* (Fares-Taie et al. 2013) described the first biallelic mutations of this gene in 3 A/M consanguineous families. The proband (II.3) from family 3 reported in this study (carrying the *ALDH1A3* splice site mutation c.475+1G>T, NM_000693) is shown in Fig. 1b. Since then, *ALDH1A3* has been implicated in many reports of recessive forms of A/M (Abouzeid et al. 2014; Alabdullatif et al. 2017; Aldahmesh et al. 2013b; Dehghani et al. 2017; Lin et al. 2018; Liu et al. 2017b; Mory et al. 2013; Roos et al. 2013; Semerci et al. 2014; Ullah et al. 2016; Yahyavi et al. 2013). Mutations in *ALDH1A3* are a frequent cause of A/M in consanguineous pedigrees (Abouzeid et al. 2014), representing approximately 10% of cases. Of note, mutations in *ALDH1A3* have been reported mainly in consanguineous pedigrees. Different types of

mutations have been described (missense, nonsense and splice site variants) and appear to act through a “loss-of-function” mechanism. The ocular defects are generally severe (anophthalmia or severe microphthalmia) and bilateral. For patients also presenting with extraocular findings, neurocognitive and behavioral features are quite frequent, notably the presence of autistic features. There are also rare descriptions of systemic anomalies, such as Dandy Walker malformation (Semerci et al. 2014), pulmonary stenosis and atrial septal defects (Fares-Taie et al. 2013). Incomplete penetrance of biallelic *ALDH1A3* mutations has been reported in one family (Plaisancie et al. 2016a).

***MAB21L2* (*male abnormal gene family 21, C. elegans, homolog-like 2*)**

In 2014, a WES study identified 4 different missense variants in *MAB21L2* in 5 unrelated families with bilateral clinical anophthalmia or microphthalmia and coloboma, with or without rhizomelic skeletal dysplasia and learning disability (Rainger et al. 2014). The first family had autosomal dominant bilateral colobomatous microphthalmia, and carried a heterozygous *MAB21L2* mutation (c.152G>A p.[Arg51His], NM_006439) that segregated with the disease. The other *MAB21L2* heterozygous variants identified by this study were found in 3 sporadic cases. Among these, one patient carried a *de novo* variant (c.151C>T p.[Arg51Cys], NM_006439) and presented with bilateral anophthalmia, macrocephaly, intellectual disability and generalized skeletal dysplasia. The same variant was also found *de novo* in a second unrelated patient with more severe rhizomelic skeletal dysplasia associated with bilateral anophthalmia. A third individual, who had bilateral colobomatous microphthalmia and minor skeletal anomalies, harbored a heterozygous variant (c.145G>A p.[Glu49Lys], NM_006439) for which segregation analysis was not possible. Finally, they also identified 2 consanguineous sibs with bilateral retinal coloboma, one with additional unilateral microphthalmia, whose asymptomatic parents were both carriers. Both siblings had a homozygous missense mutation, located in a different domain of *MAB21L2* (c.740G>A p.[Arg247Gln], NM_006439). Their parents were heterozygous and asymptomatic, indicating an autosomal recessive mode of inheritance. Interestingly, the authors noted that these two homozygous siblings were more mildly affected than individuals with autosomal dominant mutations of the gene. Therefore, they suggested that the monoallelic mutations may act via a “dominant negative” mechanism (Rainger et al. 2014). A recent study analyzing the effect of these missense variants using the 3D structure of MAB21L1, another MAB21 protein, showed how all the missense mutations reported by Rainger *et al.* (Rainger et al. 2014) are predicted to destabilize the protein (de Oliveira Mann et al. 2016). Horn (Horn et al. 2015) described in more detail the skeletal involvement in patients with the p.(Arg51Cys) variant, consisting of severe, pre- and postnatal short stature, rhizomelic limbs with a specific humero-femoral dysplasia and multiple joint contractures. Another interesting observation is that the three monoallelic variants identified by Rainger *et al.* affected neighbouring residues (p.Glu49 and p.Arg51). Another study (Deml et al. 2015) has reported a third heterozygous missense mutation affecting arginine 51 (c.151C>G p.[Arg51Gly], NM_006439), identified in a 3-generation family with coloboma, microcornea, cataracts and skeletal dysplasia. This

variant segregated with the disease and was not found in public variant databases. Taken together, these data show that the region of the protein where these monoallelic missense variants occur seems to be a mutational hotspot which may have a crucial role for the function of the protein in eye and skeletal development.

Recently Patel and colleagues (Patel et al. 2018) reported the first heterozygous *MAB21L2* nonsense mutation (c.840C>G p.[Tyr280Ter], NM_006439) in a patient with isolated colobomatous microphthalmia. Unfortunately, the segregation pattern for this sporadic case is unknown. The mechanism by which MAB21L2 exerts its effects is still unknown and the identification of additional mutations will help to elucidate this.

MAB21L2 is a member of the gene family Male-abnormal 21 and is able to antagonize BMP4 signaling which is known to be important in eye development (Bakrania et al. 2008), in particular via interaction with SMAD1 (Baldessari et al. 2004). Work in several species suggests that the Mab-21 genes play key roles in early developmental processes such as gastrulation, neural tube closure and eye formation (Chow et al. 1995; Kudoh and Dawid 2001; Lau et al. 2001; Wong and Chow 2002; Yamada et al. 2003).

Deml et al. (2015) generated zebrafish models homozygous for a frameshift truncating mutation and an in-frame deletion in *mab21l2*. The phenotype of the former included microphthalmia with small or absent lenses in all embryos, and coloboma and shortened body/curved tail in 76% and 56% of fish, respectively. For the homozygous in-frame deletion mutants, the abnormal phenotype was milder and consisted of severe ocular coloboma in all embryos and corneal defects in almost half of them, while lenses and eye size appeared to be only mildly affected. Finally, wildtype, but not mutant, *MAB21L2* mRNA was able to efficiently rescue the ocular anomalies present in zebrafish embryos with a homozygous *mab21l2* frameshift mutation (68% versus 14%).

***BMP7* (*bone morphogenetic protein 7*)**

BMP7 encodes a bone morphogenetic protein (BMP), a family of proteins involved in multiple processes within the cell and in the specification and patterning of the early embryo. Despite these important roles, little is known regarding the specific roles of individual BMPs in human disease due to their overlapping functions. In the mouse, *Bmp7* is expressed in the optic cup and surface ectoderm of the developing eye (Dudley and Robertson 1997) playing a crucial role in optic fissure formation and in the early steps of lens development. *Bmp7* null mice display severe eye defects, including A/M, skeletal and renal anomalies, and die shortly after birth (Dudley et al. 1995; Luo et al. 1995; Wawersik et al. 1999; Zouvelou et al. 2009). Interestingly, in the mouse the ocular defects exhibit reduced penetrance and variable expressivity, ranging from anophthalmia to normal sized eyes (Dudley et al. 1995; Luo et al. 1995; Wawersik et al. 1999) and are influenced by genetic background.

After screening 279 patients with an A/M and coloboma phenotype, Wyatt *et al.* (Wyatt et al. 2010) identified 3 cases with heterozygous *BMP7* variants of different types (frameshift, missense and Kozak sequence mutations). The phenotypes of the 3 patients were **i)** bilateral anophthalmia with various anomalies (c.513delA, NM_001719.2), **ii)** unilateral anophthalmia with AEG syndrome (c.-1G>T, NM_001719.2) and **iii)** unilateral microphthalmia, chorioretinal and optic disc coloboma with learning disability (c.593T>C, NM_001719.2). All three variants were maternally inherited, one from an unaffected mother (c.513delA, NM_001719.2) and the remaining two from mothers with no documented phenotype. As the segregation of these alleles is not fully known their significance may become clearer as more data is generated for this gene.

***GDF3 and GDF6* (*growth differentiation factor 3 and 6*)**

The Growth Differentiation Factors (GDFs) are members of the BMP sub-family of transforming growth factor-beta (TGF-β) signaling ligands, known to regulate patterning during development (Herpin et al. 2004).

All of the pathogenic variants reported to date for *GDF3* and *GDF6* are heterozygous missense changes with affected individuals exhibiting either ocular or skeletal anomalies, or a combination of the two. Variants in these genes account for 1.7% and 1% of A/M and coloboma patients screened for *GDF3* or *GDF6* mutations, respectively (Asai-Coakwell et al. 2007; Asai-Coakwell et al. 2009; Ye et al. 2010). The range of ocular phenotypes extends from bilateral anophthalmia (*GDF6* variant) to unilateral coloboma (*GDF3* variant). In addition to A/M and coloboma, potential pathogenic missense variants in *GDF6* have been recently reported in 3 unrelated patients with isolated primary glaucoma, two with dominant forms and one sporadic case (Huang et al. 2015).

Monoallelic variants in *GDF3* and *GDF6* are associated with both high intra- and interfamilial variability. For example, the *GDF3* variant reported in the case with unilateral coloboma (Ye et al. 2010) was inherited from the mother who was diagnosed with a familial Klippel-Feil syndrome (MIM#118100), a rare skeletal disorder mainly characterized by the fusion of cervical vertebrae. An example of interfamilial variability is provided by a *de novo* missense variant in *GDF6* identified in one patient with bilateral anophthalmia, but also in 3 unrelated cases with coloboma, microphthalmia, post-axial polydactyly and Klippel-Feil syndrome (Asai-Coakwell et al. 2009). Beside this clinical variability, non-penetrance also seems to be a reasonably common occurrence associated with *GDF3* and *GDF6* variants (Asai-Coakwell et al. 2009; Bardakjian et al. 2017; Patel et al. 2018; Ye et al. 2010). This relatively frequent non-penetrance could be due to an additive effect of multiple variants in several BMP ligands contributing to the ocular or skeletal phenotypes (Ye et al. 2010). For instance, a patient with severe microphthalmia, bilateral iris coloboma and optic nerve hypoplasia had both *GDF3* and *GDF6* heterozygous variants, with only the former being inherited from their affected mother, who

presented with mild microphthalmia, iris coloboma and normal optic discs [Ye et al. 2010]. Documented skeletal features associated with mutations in these genes include post-axial polydactyly, hemifacial hypoplasia, hemi-vertebrae, rib anomalies and spondylothoracic dysostosis (Asai-Coakwell et al. 2009). Additional features that have been observed include orofacial clefting, small pituitary gland with growth hormone deficiency, hydrocephalus and talipes (den Hollander et al. 2010).

***VAX1* (*ventral anterior homeobox 1*)**

VAX1 is a homeodomain transcription factor important for eye and brain development and its role has been studied in several animal models (Bertuzzi et al. 1999; Hallonet et al. 1999; Take-uchi et al. 2003). Mice lacking *Vax1* died perinatally, although some survived after birth (Bertuzzi et al. 1999; Hallonet et al. 1999), exhibiting cleft palate and coloboma due to the failure of optic fissure closure. In addition, these animals were unable to develop the optic chiasm and presented with axon guidance defects in the form of retinal ganglion cells whose axons were unable to properly localize within the brain (Kim et al. 2014). Kim et al. (2014) suggested that, in addition to its function as a transcription factor, Vax1 may also work as a secreted protein to promote the growth of retinal ganglion cells towards the brain. To date, only one patient has been reported whose A/M phenotype is due to an alteration of VAX1 (Slavotinek et al. 2012). The patient, identified during the screening of 70 cases with an A/M phenotype, had bilateral severe microphthalmia and optic nerve hypoplasia, global developmental delay, hippocampal anomalies, agenesis of the pineal gland and corpus callosum, growth retardation and bilateral cleft lip and palate. This individual carried two adjacent homozygous substitutions (c.453G>A and c.454C>A, NM_001112704.1) resulting in a missense change located in the VAX1 homeodomain (p.[Arg152Ser]). Both parents were unaffected carriers of the variant.

<u>Colobomatous microphthalmia</u>

Simple microphthalmia refers to an anatomically intact eye with a reduced axial length, without abnormalities of the anterior or posterior segments. However, this ocular growth restriction is frequently associated with optic fissure closure defects. The question of whether optic fissure closure defects are the consequence or the cause of abnormalities in ocular growth and differentiation/specification remains unknown, making the classification of microphthalmia and coloboma genes according to mechanism challenging. Therefore, the genes described in the following section are those that have been linked to ocular growth defects through identification of mutations in patients with simple microphthalmia and/or coloboma, although the distinction may be somewhat arbitrary.

***TENM3* (*teneurin transmembrane protein 3*)**

TENM3 is a member of the Tenm/Odz (teneurin) family, which comprises four type II transmembrane molecules. These proteins consist of at least five functional units: a N-terminal cytoplasmic region, a transmembrane domain, a linker region, a dimerization (EGF) unit and a large globular C-terminal domain (Feng et al. 2002). In addition to their transmembrane role, it has been shown that the intracellular domains of at least two teneurins can undergo proteolytic cleavage and translocate to the nucleus where they regulate transcriptional activity (Young and Leamey 2009). Genetic studies of fly Tenm/Odz demonstrate a crucial role of teneurin during segmentation. Furthermore, both *in vitro* and *in vivo* studies suggest that teneurins are able to promote neurite outgrowth and cell adhesion (Young and Leamey 2009). Moreover, multiple lines of evidence support the involvement of TENM3 in vertebrate eye development. It is expressed multiple regions of the developing eye, including the optic stalk (Ben-Zur et al. 2000). Knockout mouse models have significantly impaired binocular vision as a result of abnormalities in the mapping of ipsilateral projections in the optic pathway (Leamey et al. 2007). Moreover, zebrafish *tenm3* knockdown models have phenotypes including errors of both stratification and targeting of dendrites and axons within a subset of retinal ganglion cells (RGCs). This indicates that the gene plays a role in wiring subsets of functionally defined visual circuits (Antinucci et al. 2013). However, it is important to note that none of these animal models presented with overt ocular malformations.

By combining linkage and WES analysis, a homozygous mutation (c.2083dup p.[Thr695Asnfs] NM_001080477) in *TENM3* (historically *ODZ3*) was identified in two children with isolated colobomatous microphthalmia from a consanguineous family (Aldahmesh et al. 2012). More recently, we and others (Chassaing et al. 2016b; Patel et al. 2018) confirmed the involvement of this gene in autosomal recessive colobomatous microphthalmia by describing consanguineous families in whom genetic studies identified *TENM3* homozygous mutations, including a splicing variant (c.2968-2A>T, NM_001080477) and the frameshift variant reported by Aldahmesh *et al*. (2012).

***C12orf57* (*chromosome 12 open reading frame 57*)**

Although the structure and function of its product are not known, biallelic variants in *C12orf57* have been identified in familial and sporadic cases of microphthalmia with bilateral iris and/or chorioretinal coloboma (Patel et al. 2018; Platzer et al. 2014; Zahrani et al. 2013), unilateral iris or chorioretinal coloboma and posterior staphyloma (Salih et al. 2013), and microcornea with corneal opacity and dense cataract (Salih et al. 2013). Additional neurologic features have been observed, such as global developmental delay and epilepsy, corpus callosum anomalies and behavioral difficulties, consistent with Temtamy syndrome (MIM#218340). Temtamy syndrome is a rare neurological disorder characterized by agenesis/hypoplasia of corpus callosum with developmental abnormalities that

includes ocular, skeletal and craniofacial abnormalities (Temtamy et al. 1996). In a cohort of 27 consanguineous families with corpus callosum hypoplasia, *C12orf57* screening revealed 10 patients from 4 families homozygous for the same variant (c.1A>G p.[Met1?], NM_138425) affecting the initiator methionine codon (Akizu et al. 2013). Haplotype analysis was consistent with a founder effect. Two out of the 10 patients had visual abnormalities (optic disc atrophy and colobomatous microphthalmia). A recent review of 56 cases with Temtamy syndrome carrying biallelic *C12orf57* mutations (23 previously unpublished cases and 33 cases from the literature) has shown that microphthalmia was present in 16.4% of the cases and coloboma in 14.5% of the cases (Alrakaf et al. 2018).

***YAP1* (*yes associated protein 1*)**

YAP1 encodes a transcriptional coactivator that represents one of the major effectors of the Hippo pathway, and plays a role in development, growth, repair and homeostasis. It is also noteworthy that *SOX2* is able to directly regulate *YAP1* (Seo et al. 2013). YAP1 protein has an AKT phosphorylation/14-3-3-binding site, followed by a WW domain, a transcription activation domain and a C-terminal PDZ-binding motif (Komuro et al. 2003).

In affected members of two unrelated families with optic fissure closure defects, Williamson *et al.* (Williamson et al. 2014) identified two heterozygous nonsense mutations in *YAP1*. The first mutation (c.370C>T p.[Arg124Ter], NM_001130145.2) was present in a family (4 individuals) with isolated bilateral ocular coloboma. The second variant (c.1066G>T p.[Glu356Ter], NM_001130145.2), was identified in a family previously reported by Ravine *et al.* (Ravine et al. 1997) with microphthalmia ranging from mild to severe bilateral, iris and/or chorioretinal coloboma and extraocular anomalies including hearing loss, intellectual disability, orofacial clefting and hematuria. The presence of an alternative transcription start site (TSS) in intron 1 of *YAP1* may explain the more restricted phenotype in the first family. The p.(Glu356Ter) mutation in the family with the syndromic phenotype is present in the coding sequence of all transcripts from either the canonical or alternative TSS. In contrast, the p.(Arg124Ter) mutation in the family with isolated ocular coloboma is located in the 5' UTR of the alternative transcript. Therefore, according to the authors, this phenotypic difference might be explained by a partial rescue of *YAP1* haploinsufficiency by alternative transcripts, which they supported by demonstrating that both *YAP1* TSSs were used in every developmental and adult tissue examined (human fetal brain and mouse embryonic brain, eye, kidney, whole embryo, and various adult tissues).

In addition, Holt *et al.* (Holt et al. 2017) screened *YAP1* for variants in a cohort of 258 patients with A/M and coloboma phenotypes and identified a frameshift mutation (c.1160delA p.[Asn387ThrfsTer16], NM_001130145.2) in a patient with bilateral microphthalmia with chorioretinal

coloboma and Asperger's syndrome (Fig. 2). This variant is located in the coding region (exon 7) of all *YAP1* transcripts and is predicted to result in the loss of the transactivation domain. However, sequencing of cDNA from the patient showed the variant does not result in nonsense mediated decay. This novel frameshift mutation was also inherited from an asymptomatic parent. Several hypotheses for the difference in phenotype between the patient and parent can be proposed, including incomplete penetrance, modifying mutations in the index case or parental mosaicism. Furthermore, Oatts *et al*. (Oatts et al. 2017) reported a novel heterozygous missense mutation (c.284T>C p.[Phe95Ser], NM_001130145.2) in a family with dominant isolated ocular coloboma and evidence of incomplete penetrance.

Further evidence supporting the role of *YAP1* in human ocular defects is provided by zebrafish *yap1* mutants, which exhibit coloboma and loss of retinal pigment epithelium in a fully penetrant fashion, but with varying extent and localization (Miesfeld et al. 2015).

***SALL2* (*spalt like transcription factor 2*)**

SALL2 is a member of the spalt-like family of zinc finger transcription factors. Humans have 4 spalt-like genes, each with one N-terminal C2HC Zinc finger and 3 or 4 C2H2 Zinc finger domains for interactions with DNA or proteins. *SALL2* appears to be mammalian-specific. Kelberman *et al*. (Kelberman et al. 2014) identified a homozygous nonsense mutation in *SALL2* in a consanguineous family with isolated iris and retinal coloboma. They subsequently generated *Sall2*-null mice and, although no overt phenotypic abnormalities were observed, histological analysis of the eyes revealed a colobomatous phenotype, but with no evidence of microphthalmia. Of note, Ullah *et al.* (Ullah et al. 2017) recently identified by WES two missense variants in *SALL4*, another member of the spalt-like genes family, inherited *in trans* in a patient with unilateral microphthalmia and coloboma, bilateral optic nerve hypoplasia, cardiac defects and growth delay. Haploinsufficiency of *SALL4* as a result of truncating mutations is linked to acro-reno-ocular syndrome (Okihiro syndrome, Duane-radial ray syndrome) (MIM#607323), the features of which include Duane syndrome, eye anomalies, as well as radial ray and renal anomalies (Kohlhase et al. 2005).

***ABCB6* (*ATP binding cassette subfamily B member 6*)**

ABCB6 codes for a protein belonging to the B subfamily of ATP-binding cassette (ABC) transporters. Heterozygous variants in *ABCB6* were first identified in a Chinese three generation family with autosomal dominant isolated iris and chorioretinal coloboma, where a *ABCB6* missense variant (c.2431C>G p.[Leu811Val], NM_005689) was found to segregate with the phenotype (Wang et al. 2012). Following this, Wang *et al.* (Wang et al. 2012) also identified another heterozygous variant (c.169G>A p.[Ala57Thr], NM_005689) in three Indian sporadic cases of colobomatous microphthalmia

with no parental data available. The same study showed that a morpholino knockdown of *abcb6* in zebrafish resulted in coloboma and delayed development. These phenotypes could be rescued using wildtype *ABCB6* mRNA, but not by injection of human mRNA containing the mutations identified in their patients. Prokudin *et al.* (Prokudin et al. 2014) also later reported an *ABCB6* heterozygous variant in a patient with bilateral iris and fundus coloboma inherited from the unaffected father. In addition, heterozygous *ABCB6* nonsynonymous variants have been identified in familial and sporadic cases with dyschromatosis universalis hereditaria (MIM#615402) (Cui et al. 2013; Liu et al. 2014; Zhang et al. 2013). In humans, skin tissue and melanocytes express *ABCB6* (Zhang et al. 2013). Knockdown of the gene in zebrafish resulted in the reduction of the number of mature melanocytes, supporting its role in dyschromatosis universalis hereditaria. Liu *et al.* (Liu et al. 2014) apparently found ocular anomalies in some patients with this condition, although the clinical description ('abnormal pitting lack of iris' (sic)) is not clear, and no pictures are available. Therefore, these reports may highlight common mechanisms underlying both pigmentary dyschromatosis universalis hereditaria and ocular coloboma.

Finally, several individuals with the Langereis (-) blood group phenotype (MIM#111600) were found to have biallelic truncating and missense mutations in *ABCB6*. However, there were no reports of coloboma or eye defects in these individuals (Helias et al. 2012; Saison et al. 2013).

Complex microphthalmia and aphakia

Complex microphthalmia refers to an eye with a reduced axial length and abnormalities of the anterior and/or posterior segment of the eye. As with previous sections, the following describes the genes implicated in this phenotype through identification in cases with complex microphthalmia. This section includes genes responsible for aniridia, anterior segment dysgenesis and aphakia.

***PAX6* (*paired box 6*)**

PAX6 is one of the nine members of the paired box gene family, the primary function of which is to regulate transcription in order to promote correct brain and eye morphogenesis (Hever et al. 2006). It was the first gene to be associated with A/M in a patient who had compound heterozygous mutations and severe eye and brain anomalies (Glaser et al. 1994). However, individuals with compound heterozygous mutations of *PAX6* rarely survive due to the severity of their brain anomalies (Glaser et al. 1994; Schmidt-Sidor et al. 2009; Solomon et al. 2009) and those with heterozygous mutations only rarely display A/M (Chassaing et al. 2014; Dansault et al. 2007; Hever et al. 2006). In our cohort, we recently identified a *de novo* heterozygous *PAX6* missense variant (c.160A>C p.[Ser54Arg], NM_000280) in a boy with clinical anophthalmia (unpublished data, Fig. 3a). The typical phenotype associated with heterozygous variants of this gene is aniridia (absence of the iris) with associated

manifestations in other ocular structures, for example foveal hypoplasia, and corneal opacification (Hall et al. 2018). Atypical presentations include Peters' anomaly, corectopia, isolated foveal hypoplasia, optic nerve malformations (hypoplasia and coloboma), cataract and nystagmus (usually related to reduced visual function) (Hever et al. 2006; Tzoulaki et al. 2005). In total, more than 500 different mutations have been described (HGMD®) including amino acid substitutions, premature termination codons or C-terminal extension, splicing alterations either in coding or non-coding exons (Hingorani et al. 2012; Plaisancie et al. 2018b). In addition to point mutations, chromosome 11p13 deletions have also been described in patients with aniridia. These can include the entirety of *PAX6* and neighbouring genes or can just affect the downstream 3' regulatory region essential for *PAX6* expression and act through a "position effect" (Ansari et al. 2016; Fantes et al. 1995). Deletions spanning *PAX6* and *WT1* are the cause of a contiguous gene syndrome that includes Wilms tumor, Aniridia, Genital abnormalities and mental Retardation (WAGR syndrome, OMIM #194072).

Transmission of *PAX6* mutations is autosomal dominant with strong penetrance, but variable expressivity. In about one third of the cases the mutations occur *de novo* (Vincent et al. 2003).

FOXE3 (forkhead box E3)

FOXE3 is a single-exon gene encoding a transcription factor of 319 amino acids with a DNA-binding domain, called forkhead domain, located from amino acid 71 to 165 (Ormestad et al. 2002). *FOXE3* is specifically expressed during lens development in humans, mouse and zebrafish (Blixt et al. 2000; Iseri et al. 2009; Semina et al. 2001; Shi et al. 2006). It participates in lens vesicle formation and allows the maintenance of the lens cells in a proliferative state, thus preventing their early differentiation into lens fiber cells (Blixt et al. 2000; Landgren et al. 2008). *FOXE3* is known to be one of the *PAX6* master gene targets (Dimanlig et al. 2001) and recently, Khan *et al.* (Khan et al. 2016) demonstrated that its function is mediated via transcriptional regulation of *DNAJB1*, which is vital for development of the lens and the maintenance of its transparency. We and others have shown that truncating and missense *FOXE3* mutations are involved in a recessive phenotype of primary congenital aphakia, microphthalmia and sclerocornea (Ali et al. 2010; Anjum et al. 2010; Chassaing et al. 2014; Garcia-Montalvo et al. 2014; Iseri et al. 2009; Islam et al. 2015; Jimenez et al. 2011; Khan et al. 2016; Pantoja-Melendez et al. 2013; Plaisancie et al. 2018a; Reis et al. 2010; Saboo et al. 2017; Ullah et al. 2016; Valleix et al. 2006). Dominant forms of variable ocular anomalies, including anterior segment dysgenesis, blue dot lens opacities and microphthalmia, have been reported with heterozygous *FOXE3* mutations (Bremond-Gignac et al. 2010; Doucette et al. 2011; Iseri et al. 2009; Semina et al. 2001). In a recent review, we show correlations between mutation type, inheritance pattern and the severity of the phenotype (Plaisancie et al. 2018a). Complex microphthalmia and/or primary aphakia is present in all patients carrying biallelic *FOXE3* mutations with at least one truncating mutation, except for one familial case

of Peters' anomaly (Khan et al. 2016). Extraocular features, such as global developmental delay and various malformations (Arnold-Chiari malformation, ventricular septal defect, polycystic ovary syndrome, hypertrichosis) have been variably described in individuals with truncating mutations. Isolated severe ocular phenotypes (microphthalmia and/or primary aphakia) are also observed in around 75% patients with biallelic missense mutations. Dominant forms are associated with the presence of mutations leading to a C-terminal extension of the protein and are responsible for isolated anterior segment dysgenesis, sclerocornea and congenital cataract (Plaisancie et al. 2018a).

PITX3 (paired like homeodomain 3)

The pituitary homeobox (PITX) family of genes encodes transcription factors that contain two functional domains, the homeobox and the OAR (otp, aristaless, rax), that function in DNA-binding and potentially transactivation, respectively. Individuals with pathogenic heterozygous variants in *PITX3* manifest isolated and syndromic congenital cataract, anterior segment disorders, such as Peters' anomaly, posterior embryotoxon and sclerocornea (Aldahmesh et al. 2011a; Berry et al. 2004; Bidinost et al. 2006; Burdon et al. 2006; Finzi et al. 2005; Liu et al. 2017a; Semina et al. 1998; Summers et al. 2008; Verdin et al. 2014). Of note, one patient has been reported with a heterozygous variant in *PITX3* and complex microphthalmia associated with autism and teeth anomalies (Zazo Seco et al. 2018). Most reported mutations are frameshifts, except for one apparently recurrent missense mutation in exon 2 (c.38G>A p.[Ser13Asn], NM_005029.3) in a patient with congenital cataract (Semina et al. 1998) and in a dominant family with Peters' anomaly (Zazo Seco et al. 2018). Interestingly, all the frameshift variants reported to date are located in exon 4 (upstream from the OAR domain) and none so far within the homeodomain or the OAR domain-coding regions. Of note, homozygous frameshift mutations of *PITX3* have been identified in four consanguineous individuals (Aldahmesh et al. 2011a; Bidinost et al. 2006; Zazo Seco et al. 2018). Three of them presented with a more severe ocular phenotype (complex microphthalmia) than the heterozygous family members (from no ocular phenotype to cataract) (Aldahmesh et al. 2011a; Bidinost et al. 2006). The two affected siblings with homozygous variants reported by Bidinost *et al.* (Bidinost et al. 2006) had additional neurological features characterized by intellectual deficiency, choreiform movements and increased muscle tone, and decreased deep tendon reflexes of the lower extremities.

***PTCH1* (*patched, drosophila, homolog of, 1*)**

PTCH1 is a transmembrane receptor for the secreted hedgehog ligands (SHH, IHH, DHH), which functions as part of a dosage-sensitive pathway resulting in the activation of downstream target genes. Hedgehog signaling is important in embryonic development and tumorigenesis. Heterozygous

mutations of this gene have been associated with basal cell nevus syndrome, also called Gorlin syndrome (MIM#109400) and holoprosencephaly (Ming et al. 2002).

Eye anomalies have occasionally been reported in patients with Gorlin syndrome. However, attention was drawn to the occurrence of severe eye anomalies in one patient with unilateral microphthalmia with orbital cyst who developed a medulloblastoma and was subsequently diagnosed with a *PTCH1* mutation (c.1402delG, NM_000264), confirming a diagnosis of Gorlin syndrome (Ragge et al. 2005c).

A recent study has shown that *PTCH1* seems also to be a more significant contributor to congenital ocular malformations, given the identification of likely pathogenic heterozygous *PTCH1* variants in 10% of the cohort (Chassaing et al. 2016a). First, in a cohort of 22 patients with various ocular developmental anomalies, Chassaing *et al.* identified 4 unrelated patients with heterozygous variants predicted to be deleterious by *in silico* analysis: one patient (P5) with microphthalmia, cataract and sclerocornea had a frameshift deletion (c.4delG p.[Glu2AsnfsTer9], NM_001083603), one patient (P20) with bilateral Peters' anomaly had a missense mutation (c.3947A>G p.[Tyr1316Cys], NM_000264) and two patients (P8 and P15) with colobomatous microphthalmia, corpus callosum abnormality and atrial septal defects had missense mutations (c.3191C>T p.[Thr1064Met] and c.3241G>A p.[Val1081Met] respectively, NM_000264). With the exception of P5, for whom the authors were unable to perform segregation analysis, the mutations were inherited from asymptomatic parents, suggesting incomplete penetrance. Screening for mutations in *PTCH1* in additional cohorts identified three further cases: i) a patient (P17) with Axenfeld-Rieger anomaly who had a heterozygous *PTCH1* missense mutation (c.3889C>T p.[Arg1297Trp], NM_000264) inherited from an asymptomatic parent, ii) a patient (CC10) with bilateral Peters' anomaly and a variant (c.2695A>G p.[Ile899Val], NM_000264) also inherited from an asymptomatic parent and iii) a patient (CC44) with a familial form of dominant A/M and anterior segment dysgenesis with a variant (c.2332A>C p.[Thr778Pro], NM_000264) for which segregation analysis has not been performed. Zebrafish models demonstrated that these missense mutations identified in humans had a deleterious effect and that *in vivo* suppression of ptch1 results in microphthalmia (Chassaing et al. 2016a).

Given the critical role of the SHH pathway in eye morphogenesis (Cavodeassi et al. 2018), *PTCH1* involvement in ocular developmental defects would not be surprising. However, there have been no other reports of *PTCH1* mutations in developmental eye anomalies. This may be due to the difficulty in interpreting the identified variants as pathogenic, since most were missense variants inherited from asymptomatic parents, requiring complex *in vivo* functional studies to validate their pathogenicity.

***MAF* (m*af bZIP transcription factor V*)**

MAF encodes a basic-leucine zipper domain (bZip) transcription factor, which acts as a homo- or hetero-dimer. Depending on the binding site and binding partner, it can function as a transcriptional activator or repressor (Kataoka 2007). Heterozygous mutations have been reported in 80 individuals who exhibit cataract of various types, but can also present with other ocular defects such as iris coloboma, glaucoma, microcornea, Peters' anomaly and microphthalmia (Anand et al. 2018). Heterozygous mutations in *MAF* can also be associated with Ayme-Gripp syndrome (MIM#601088), which presents with congenital cataract, craniofacial features, brachycephaly, deafness, intellectual disability and seizures (Niceta et al. 2015). With the exception of one translocation event (Jamieson et al. 2002), all reported *MAF* mutations are missense changes. A genotype-phenotype correlation appears to exist since a significant proportion of the mutations associated with eye defects are located within the basic-leucine zipper domain. In contrast, no mutations have been identified so far within the transactivation domain in humans, although such a mutation has been reported in the mouse model with congenital cataract (Perveen et al. 2007). In addition, several mutations within the N-terminal region upstream of the transactivation domain are associated with the Ayme-Gripp syndrome.

***PXDN* (*peroxidasin*)**

PXDN is a peroxidase which is secreted into, and involved in the formation of, the extracellular matrix. In mice, peroxidasin protein (Pxdn) is localized in the corneal and lens epithelium (Khan et al. 2011). A WES study performed in a non-consanguineous family containing two siblings with anterior segment dysgenesis, sclerocornea, microphthalmia and neurodevelopmental delay identified compound heterozygous *PXDN* mutations in both sibs, with one variant inherited from each parent (Choi et al. 2015). They also reported two additional compound heterozygous *PXDN* mutations in a singleton male with bilateral microphthalmia, cataract and anterior segment dysgenesis, one inherited from each parent (Choi et al. 2015). Homozygous changes in *PXDN* have been identified in two families with non-syndromic A/M by Patel *et al*. (2018). Biallelic mutations in *PXDN* have been shown to be responsible for congenital cataracts, microcornea, sclerocornea and developmental glaucoma in three families (Khan et al. 2011) and recently for developmental glaucoma in one family (Micheal et al. 2016). A mouse model with a homozygous truncating mutation in the peroxidase domain demonstrated severe anterior segment dysgenesis and microphthalmia, resembling the phenotypes of patients (Yan et al. 2014).

***GJA8* (*gap junction protein alpha 8*)**

GJA8 encodes gap junction protein alpha 8, also known as connexin 50 (Cx50). Connexins are transmembrane proteins that oligomerise to form gap junction channels (GJCs), which mediate the passage of ions and small molecules between adjacent cells and therefore play an important role in intercellular communications. The first link between *GJA8* and cataractogenesis emerged from linkage analysis of an 8-generation family diagnosed with zonular pulverulent cataract (Renwick and Lawler 1963; Shiels et al. 1998). Since then, *GJA8* has been investigated mainly in individuals with congenital or early-onset cataracts. These studies have progressively expanded the spectrum of *GJA8* variants associated with these developmental anomalies.

Although most individuals initially described with *GJA8* variants had isolated cataract, a recent study (Ma et al. 2016) identified a *de novo* heterozygous missense mutation (c.151G>A p.[Asp51Asn], NM_005267.4) in a patient with a more severe phenotype, including microphthalmia and sclerocornea. To investigate further the role of *GJA8* in A/M, Ceroni *et al.* (Ceroni et al. 2018) analyzed a large cohort of individuals presenting with a wide range of developmental eye anomalies, mainly A/M and coloboma. This study identified 6 likely pathogenic sequence variants in 7 independent families, including the variant previously reported by Ma *et al*. (Ma et al. 2016). Interestingly, in all 6 families where segregation analysis could be performed, these variants co-segregated with both early onset cataracts and microphthalmia, supporting the hypothesis that the gene may be involved a broader range of human developmental eye anomalies. The missense variant p.(Gly94Arg) (c.280G>A, NM_005267.4) for which segregation analysis could not be performed was identified in a child with congenital aphakia, sclerocornea, microphthalmia and coloboma (Ceroni et al. 2018) (Fig. 3b,c). This, in combination with two recently reported *de novo* mutations involving the same amino acid (c.280G>C p.[Gly94Arg] and c.281G>A p.[Gly94Glu], NM_005267.4), in singleton cases with severe lens developmental abnormalities, sclerocornea and microcornea (Ma et al. 2018), supports a more fundamental role for *GJA8* in human lens development. Moreover, these findings suggest that specific variants in *GJA8* give rise to phenotypes overlapping with those caused by genetic variants of *FOXE3* (Plaisancie et al. 2018a).

The involvement of *GJA8* in lens and ocular growth is also supported by animal studies. Mice with *Gja8* homozygous deletions or heterozygous missense mutations exhibit microphthalmia, smaller lenses, and cataract, indicating that this connexin is important not only for lens formation and the maintenance of its transparency, but also for the control of eye morphogenesis. Taken together, these studies underline the importance of screening *GJA8* more widely and in particular in individuals diagnosed with cataracts and microphthalmia or with lens defects.

In humans, *GJA8* mutations are predominantly heterozygous missense variants, with the exception being a few homozygous variants (mainly frameshift) reported in consanguineous families. Therefore, the mode of inheritance is typically autosomal dominant and this should be taken into account when providing genetic counselling.

***SIX6* (*sine oculis homeobox 6*)**

SIX6 belongs to the sine oculis homeobox (SIX) protein family, a group of evolutionarily conserved transcription factors (Kumar 2009). In *Drosophila* this family includes three genes, two of which (*sine oculis* and *optix*) are required for the correct development of the visual system (Cheyette et al. 1994; Serikaku and O'Tousa 1994). *SIX6* was mapped to chromosome 14q22.3q23 by Gallardo *et al.* (Gallardo et al. 1999). Deletions in this region had been previously reported in three individuals with bilateral clinical anophthalmia and pituitary anomalies (Bennett et al. 1991; Elliott et al. 1993; Lemyre et al. 1998). Gallardo *et al.* (Gallardo et al. 1999) demonstrated that one of these deletions (Bennett et al. 1991) included *SIX6* and suggested that the ocular and pituitary phenotype of this individual could be caused by *SIX6* haploinsufficiency, implicating it as a candidate gene for anophthalmia (Gallardo et al. 1999). Since this original report, three additional studies have linked *SIX6* variants with developmental eye disorders. In 2004, Gallardo *et al.* (Gallardo et al. 2004) performed mutational analysis of *SIX6* in a cohort of 73 patients with A/M and identified a variant of unknown significance (c.493A>G p.[Thr165Ala], NM_007374.2) in a patient with bilateral complex microphthalmia and her asymptomatic father. Using a WES and linkage analysis strategy, Aldahmesh *et al*. (Aldahmesh et al. 2013a) identified a homozygous *SIX6* truncating mutation (c.532_536del, NM_007374.2) in two children with isolated complex microphthalmia from a consanguineous family. Most recently, Yariz *et al.* (Yariz et al. 2015) reported a consanguineous family with three children who had optic disc anomalies and macular atrophy without microphthalmia or cataracts, carrying a homozygous missense mutation (c.110T>C p.[Leu37Pro], NM_007374.2). However, in a study of 173 patients with microphthalmia, clinical anophthalmia and coloboma, Aijaz *et al.* (Aijaz et al. 2004) found no disease-causing mutations in this gene.

***ATOH7* (*atonal bHLH transcription factor 7*)**

ATOH7, the human homolog of the *Drosophila* gene *atonal,* is a single-exon gene coding for a member of the basic helix-loop-helix (bHLH) transcription factor family. The bHLH domain, which in the human protein (NP_660161.1) spans residues 41-96, consists of a basic motif (residues 41-52) necessary for DNA binding, and a HLH domain (residues 53–96) involved in the formation of homo- or hetero-dimers with other family members. In 2011, Ghiasvand *et al*. (Ghiasvand et al. 2011) identified a 6,523 bp deletion located ~20 kb from *ATOH7* and affecting *cis*-regulatory elements in a large consanguineous Kurdish pedigree with autosomal recessive non-syndromic congenital retinal non-attachment (NCRNA). Subsequently, homozygous mutations affecting *ATOH7* have been identified in four additional families with NCRNA, one reported by Kondo *et al.* (Kondo et al. 2016) and three unrelated Pakistani families reported by Keser *et al.* (Keser et al. 2017), two of which carried the previously described 6,523 bp deletion. Using WES and subsequent candidate-gene screening, homozygous variants in *ATOH7* (c.146A>T p.[Glu49Val] and c.53delC p.[Pro18ArgfsTer69], NM_145178.3) have also

been identified in two consanguineous families (Pakistani and Turkish, respectively) diagnosed with microphthalmia, microcornea, corneal opacity, nystagmus and vitreoretinal dysplasia (Khan et al. 2012). The family carrying the frameshift mutation also presented with congenital cataracts, retinal detachments, hypoplasia of the optic nerves, vitreous and retinal calcifications, and persistent fetal vasculature. A homozygous mutation (c.136A>C p.[Asn46His], NM_145178.3) associated with persistent fetal vasculature has also been identified in a consanguineous Pakistani family with three affected individuals (Prasov et al. 2012).

Overall, the phenotype of individuals carrying *ATOH7* mutations seems to be consistent with a role in retinal neurogenesis. Expression studies and animal models have shown that *Atoh7* is important for retinal ganglion cell (RGC) formation (Brown et al. 2001; Wang et al. 2001) and retinal vascular development (Edwards et al. 2012). Mice lacking *Atoh7* (formerly *Math5*) (Brown et al. 2001) develop normal sized eyes, but exhibit a dramatic reduction (>95%) in the number of RGCs and no optic nerve or chiasm. However, this does not exclude that mutations of specific residues might result in other severe phenotypes, as shown by Khan *et al*. (Khan et al. 2012). Therefore, the potential involvement of *ATOH7* in microphthalmia warrants further investigation.

***RBP4* (*retinol binding protein 4*)**

Vitamin A (retinol) is a liposoluble substrate necessary for the synthesis of Retinoic Acid (RA), a signaling molecule with a pivotal role in eye morphogenesis. Retinol circulates in the blood bound to a carrier, called retinol binding protein 4 (RBP4), which together with thyroxine-binding transthyretin (TTR) mediates retinol transport from its main store site, the liver, to the peripheral target tissues (Cunningham and Duester 2015; Kawaguchi et al. 2007). In most animals, retinol cannot be synthesized *de novo*, but is assimilated with the diet; during gestation maternal retinol is the main retinoid source for embryos of placental species. Maternal RBP4 cannot cross the maternal-fetal interface, but retinol can diffuse across the yolk sac and placenta and bind to the zygotically synthesized RBP4 (Ward et al. 1997; Ward and Morriss-Kay 1997). Once retinol-RBP4 reaches the target tissues, RBP4 binds to the transmembrane receptor STRA6, which then mediates the cellular uptake of retinol (Cunningham and Duester 2015; Kawaguchi et al. 2007), as discussed previously.

Biallelic mutations in *RBP4* were first identified in pedigrees with autosomal recessive retinal dystrophy and iris coloboma (Cukras et al. 2012; Seeliger et al. 1999). Later, in a 7-generation family with autosomal dominant A/M and/or coloboma, after genome-wide multipoint linkage analysis Chou *et al.* (Chou et al. 2015) identified a candidate locus on chromosome 10q23. Given the importance of vitamin A in eye development, they prioritized genes in the 10q23 critical region with roles in vitamin A metabolism and found a missense mutation (c.223G>A p.[Ala75Thr], NM_006744) in *RBP4* in affected individuals and obligate carriers. Screening of 75 additional A/M/coloboma cases revealed another heterozygous missense mutation (c.217G>A p.[Ala73Thr], NM_006744) in two unrelated

patients (Chou et al. 2015). Functional analysis demonstrated that both these mutant retinol-binding proteins bind the STRA6 receptor with much higher affinity than the wild-type, yet carry little or no vitamin A. Consistent with these findings, all the p.(Ala73Thr) heterozygous carriers who were tested had fasting serum vitamin A levels below normal limits and plasma retinol fluorescence was also reduced. These mutations were hypothesized to have a dominant-negative effect and to compete with the wild-type copies of the protein. Maternal penetrance was significantly greater than paternal penetrance. Chou *et al.* (Chou et al. 2015) therefore suggested that when the *RBP4* mutation is maternally transmitted, there is decreased vitamin A delivery both at the placenta, involving maternal-derived RBP4, and later at the developing eye primordia, involving fetal-derived RBP4.

Recently, the role of *RBP4* in A/M was further supported by the identification of a heterozygous missense mutation (c.394T>A p.[Tyr132Asn], NM_006744) in a proband with an isolated bilateral complex microphthalmia phenotype (Riera et al. 2017). Moreover, a WES study has identified a homozygous mutation predicted to affect *RBP4* splicing (c.248+1G>A, NM_006744) in a patient with unilateral microphthalmia, retinal dystrophy and bilateral iris and retinal coloboma (Khan et al. 2017).

***BMP4* (*bone morphogenetic protein 4*)**

Similar to *BMP7*, *BMP4* encodes a member the TGF-beta superfamily of proteins. Members of this family act as ligands bind TGF-beta receptors causing the recruitment and activation of transcription factors resulting in gene regulation. Mutations affecting *BMP4* are associated with ocular, digital and brain anomalies. However, large deletions spanning *BMP4* necessitate careful delineation, since in some cases they can represent 14q22 microdeletion syndrome, a contiguous gene deletion syndrome that can extend to include *OTX2* (Bakrania et al. 2008). Since heterozygous *OTX2* mutations can also be responsible for A/M, when both genes are involved in the deletion it is difficult to establish the contribution of each haploinsufficient gene to the phenotype. *De novo* deletions of *BMP4* not including *OTX2* have been reported in two patients. These individuals lacked a A/M phenotype, instead presenting with either an isolated ocular phenotype of Rieger anomaly, microcornea, nystagmus and glaucoma (Reis et al. 2011b) or a phenotype that included sclerocornea, corneal opacity, glaucoma, brain anomalies and postaxial polysyndactyly (Hayashi et al. 2008).

Intragenic mutations in *BMP4* have been described in five families with an A/M phenotype, usually complex associating with anterior and/or posterior segment anomalies (Bakrania et al. 2008; Reis et al. 2011b; Zhang et al. 2009). However, it is important to note how variable the phenotype remains even in the case of intragenic mutations. The truncating mutation (c.226_227del p.[Ser76CysfsTer29], NM_001202.5) was identified in a proband with unilateral anophthalmia and coloboma, retinal dystrophy and a small anterior segment in the contralateral eye, along with postaxial polydactyly, structural brain anomalies and learning difficulties (Bakrania et al. 2008). This variant was also

observed in three additional family members affected with high myopia and/or polydactyly. A paternally inherited variant of unknown significance (c.278A>G p.[Glu93Gly], NM_001202.5) was identified in a proband with bilateral microphthalmia, broad hands with low-placed thumbs, brain anomalies and developmental delay, the father presenting only with mild inferior pigmentation of both retinas (Bakrania et al. 2008). However, for this proband a subsequent diagnosis of Kabuki syndrome was made as they also carried a pathogenic frameshift variant in *KMT2D* (Ragge, personal communication). A third missense variant (c.751C>T p.[His251Tyr], NM_001202.5), was detected in a proband with mild microphthalmia and anterior segment anomalies, as well as in his unaffected brother (Zhang et al. 2009). Reis *et al.* (Reis et al. 2011b) identified a nonsense mutation (c.592C>T p.[Arg198Ter], NM_001202.5) in a patient with anophthalmia, microphthalmia with sclerocornea, right-sided diaphragmatic hernia and hydrocephalus. Furthermore, they identified a frameshift mutation (c.171dupC p.[Glu58ArgfsTer17], NM_001202.5) in two half-siblings with anophthalmia/microphthalmia, discordant developmental delay, postaxial polydactyly and growth retardation, as well as in their unaffected mother. One of the affected siblings also carried an additional *BMP4* missense variant (c.362A>G p.[His121Arg], NM_001202.5). Moreover, from mutational screening of genes associated with anterior segment dysgenesis, microcornea and microphthalmia performed on 257 patients with isolated primary glaucoma, two missense variants in *BMP4* were identified (Huang et al. 2015). These were the previously reported variant (c.450C>G p.[Asn150Lys], NM_001202.5) (Weber et al. 2008), carried by a patient with primary angle−closure glaucoma (PACG), and the novel variant (c.502G>C p.[Gly168Arg], NM_001202.5), found in an individual with primary open−angle glaucoma (POAG).

Posterior microphthalmia and nanophthalmia

Nanophthalmia and posterior microphthalmia represent particular forms of microphthalmia characterized by extreme hyperopia. Nanophthalmia affects the whole globe and is associated with microcornea and susceptibility to angle closure glaucoma, whereas posterior microphthalmia only represents reduced growth of the posterior segment. A few genes have been found to be specifically involved in these subtypes of microphthalmia.

***MFRP* (*membrane frizzled-related protein*)**

This gene encodes a member of the frizzled-related protein family, containing a cysteine-rich domain (CRD) essential for Wnt binding and signaling (Katoh 2001). Mutations in this gene were first identified in the autosomal recessive retinal degeneration-6 (*rd6*) mouse model (Kameya et al. 2002; Sundin 2005). *Mfrp* was found to be primarily expressed in the eye (pigmentary retina and ciliary epithelium) and at lower levels in the brain (Kameya et al. 2002).

In humans, biallelic mutations in *MFRP* were first associated with nanophthalmia (Sundin et al. 2005). Later, additional phenotypes were also described, including posterior microphthalmia (Aldahmesh et al. 2011b; Matsushita et al. 2012; Nowilaty et al. 2013; Patel et al. 2018; Velez et al. 2017), sometimes with retinal dystrophy, foveoschisis and optic disc drusen (Ayala-Ramirez et al. 2006; Crespi et al. 2008; Mukhopadhyay et al. 2010; Wasmann et al. 2014; Zenteno et al. 2009). Isolated retinal dystrophy without the other MFRP-related ocular features has also been reported (Kannabiran et al. 2012). Interestingly, exon 5 appears to be a hotspot for *MFRP* mutations of various types (nonsense, frameshift and missense, as well as splice acceptor site variants). Recently, Collery *et al.* (Collery et al. 2016) showed that loss of *mfrp* causes nanophthalmia and hyperopia in zebrafish models.

***PRSS56* (*serine protease 56*)**

This gene encodes a peptidase S1 domain containing protein with a trypsin-like serine protease activity. Biallelic mutations in *PRSS56* (missense, nonsense and frameshift variants, as well as splice donor site variants) cause autosomal recessive posterior microphthalmia and nanophthalmia in humans (Gal et al. 2011; Nair et al. 2011; Nowilaty et al. 2013; Orr et al. 2011; Patel et al. 2018; Said et al. 2013) and posterior microphthalmia in mice (Nair et al. 2011).

***TMEM98* (*transmembrane protein 98*)**

TMEM98 was the first gene shown to cause autosomal dominant nanophthalmia (Awadalla et al. 2014). Linkage analysis of a large pedigree, followed by WES led to the identification of a missense variant (c.577G>C p.[Ala193Pro], NM_015544) in *TMEM98* segregating with the affected status. This gene, which codes for a transmembrane protein, is expressed in various ocular tissues, including sclera, iris, ciliary body, choroid and retina (Awadalla et al. 2014).

Four additional heterozygous variants in *TMEM98* were identified in a WES study of approximately 1200 samples from Chinese individuals with various forms of eye disorders (not specified) (Sun and Zhang 2015). None of these variants was detected in 288 unrelated healthy individuals (ethnicity not specified). Based on the gnomAD database, two of them (c.2T>C p.[Met1?] and c.149T>C p.[Ile50Thr],

NM_015544) are novel, while the other two (c.56C>T p.[Ser19Leu] and c.398A>C p.[Lys133Thr], NM_015544) are rare, p.(Ser19Leu) being found only in European (MAF= 0.00008513) and African (MAF = 0.00004473) individuals, while p.(Lys133Thr) is only present in East Asian individuals (MAF = 0.0002319). None of these patients had signs of nanophthalmia: the first 3 variants were identified in cases with high myopia, while the last was found in an individual with a cone-rod dystrophy. However, another study reported two additional heterozygous *TMEM98* variants, a missense (c.587A>C p.[His196Pro], NM_15544) and a 34-bp deletion at the exon4/intron4 boundary (described as NM_015544:c.694_721del, reported as NM_015544.2:c.236_263+6del34 in ClinVar), in two unrelated pedigrees with nanophthalmia, supporting the role of *TMEM98* in autosomal dominant nanophthalmia (Khorram et al. 2015).

IV - MANAGEMENT

As seen in the description of A/M, the distinction between isolated and syndromic forms is often imprecise. Systemic anomalies are present in 32-93% of A/M cases (Chambers et al. 2018; Roos et al. 2016; Shah et al. 2011a; Slavotinek 2011; Spagnolo et al. 1994; Tucker et al. 1996), presumably depending on ascertainment, and predominantly affecting brain, heart, kidneys and extremities (hands and feet). The precise combination of ocular and extraocular features associated with A/M can guide the search for genetic etiology, notwithstanding the overlap between the different syndromic forms. Given the high phenotypic variability between the different syndromes, even for variants in the same gene, it is difficult to define *a priori* a common management for all patients. Ophthalmological care aims to maximize the visual potential, prevent its deterioration and improve aesthetics through encouraging symmetrical socket growth and the use of prosthetics (Ragge et al. 2007). However, addressing systemic features and screening for potential complications, e.g. pituitary insufficiency, developmental delay, and kidney, heart or brain anomalies, is of paramount importance, meaning that multidisciplinary care is optimal (Ragge et al. 2007). Monitoring of growth, hearing, motor and cognitive development is essential in order to provide the best supportive care for the children, many of whom are severely visually impaired (Salt and Sargent 2014) and early intervention is critical to assist optimum development (Dale and Salt 2007). Furthermore, psychological support is essential to overcome the difficulties of life with a combination of visual, cognitive, motor and aesthetic issues. The initial assessment and an outline of ocular and extraocular management are defined for A/M and close liaison between various specialists is essential (Ragge et al. 2007).

In the context of microphthalmia, visual function may be present, but there may be cataract, glaucoma or refractive errors that are amenable to treatment. In the case of unilateral involvement, the healthy eye must be protected (glasses are recommended, even in the absence of refractive error). Electrodiagnostic testing can detect optic nerve dysfunction, retinal dystrophy or cortical visual impairment. Visual acuity should be closely monitored in those at risk in order to detect any loss of

vision, particularly in those at risk of a later onset retinal dystrophy (e.g. *BMP4, VSX2*). For severe anomalies associated with visual impairment, e.g. cataract, surgical treatment is offered. Chorioretinal coloboma can be associated with a small, but important risk of retinal detachment, which should be explained to the family, with a discussion of symptoms to look out for and an urgency to present to the specialist for assessment.

The size of the orbit is determined by the growth of the globe. Anophthalmia and severe microphthalmia are accompanied by a reduction in the size of the orbit, causing orbital asymmetry (Ragge et al. 2007). Occasionally, A/M is accompanied by cyst formation, and this may compensate by encouraging orbital growth. Socket expanders (if the eye is absent or virtually absent), including either hydrophilic or solid expanders can be used from birth to encourage orbital growth, and widen the palpebral fissure, enlarge the bony orbit and stretch the conjunctival cul-de-sacs. If a formed eye is present, a clear prosthesis may be fitted in front of an existing microphthalmic eye to develop the bony socket, if asymmetry is to be avoided (Ragge et al. 2007). In this way the health of the underlying eye is still observable in the very young in case of inflammation, and any risk of further loss of vision by occlusional amblyopia is reduced. A cosmetic shell can be introduced when the risk of either of these scenarios is reduced. Expansion of the orbital volume in anophthalmic or severely microphthalmic sockets can be managed by the use of solid prostheses of increasing size throughout childhood. Generally, prostheses should be updated regularly (even every few weeks/months in the early years) until adolescence, depending on progress with socket expansion and symmetry with the contralateral side. However, they can sometimes be associated with complications, including instability, difficulty with insertion or removal, or inappropriate stimulation of growth. They are generally bespoke (molded to the shape of the socket) and hand-painted to ensure the best aesthetic result. Sometimes, an orbital implant is needed in order that a smaller prosthesis can be used; an increase in socket lining to deepen the inferior palpebral fissure (from buccal mucosa, for example) might also improve stability of the prosthesis. In adulthood, ocular prostheses are worn and do not need to be changed as frequently since orbital growth is complete.

Genetic counseling

Genetic heterogeneity and variable penetrance makes genetic diagnosis and counseling challenging in these conditions. It used to be estimated that the risk of recurrence of A/M in the siblings of an affected child was 10-15% (Verma and Fitzpatrick 2007) in the absence of a genetic diagnosis. From our experience, this overall risk is probably lower than 10%; however this figure is incorrect in any individual case as it can range from zero to up to 50% depending on the situation. In the majority of cases where invasive prenatal diagnosis is not possible or desired, prenatal screening can be performed by detailed ultrasound scanning (although this forms an incomplete assessment) following by evaluation postnatally. The identification of a causal genetic anomaly (currently possible in

approximately 25-60% of cases, depending on severity, bilaterality and systemic features) enables specific genetic counseling and the opportunity for pre-natal or even pre-implantation diagnosis. However, even in families where a genetic cause is identified, genetic counseling is highly complex due to the phenotypic variability observed for many of the A/M genes (including within a family), the incomplete penetrance of mutations and the possibility of mosaicism. All these aspects and their implications in terms of genetic counseling should be discussed with the families by an ophthalmic geneticist experienced in managing these conditions.

CONCLUSION

This review reflects the wide clinical and genetic heterogeneity observed in patients with A/M, underlying the variable expressivity and incomplete penetrance that are commonly associated with these disorders. It also highlights the difficulty in establishing genotype-phenotype correlations in these conditions. The only clear conclusion, even if no statistics are yet definitive, is the more severe and bilateral the ocular lesions, the more likely it is that a causative mutation can be identified. Although hundreds of genes are implicated in eye development, we are currently able to explain the genetic bases of these defects in less than half of patients. This suggests a number of unknown genes, cellular and molecular mechanisms still remain to be elucidated in the A/M spectrum and more widely in eye development disorders. WGS technologies will provide increased opportunities for variant identification in both coding and non-coding regions of known and new A/M genes. However, along with this is the importance of multidisciplinary input from expert clinicians and scientists to interpret genetic variants in the light of the phenotype so that errors in interpretation of causality can be avoided. Nevertheless, beyond the genetic bases, parallel progress needs to be made in understanding the underlying cellular processes so as to distinguish between mechanisms that involve lack of development or regression. Indeed, in this review we have shown that some of the genes and mechanisms described provide clues indicating that many cases of microphthalmia are related to impairment of development of structures, such as lens or retina, or defects in optic fissure closure. Therefore, as in genetic sequences such as Pierre-Robin sequence, in which one morphogenetic cause triggers a cascade of effects, reduction in eye size may be more the consequence of a particular ocular structure development defect (lens, retina, optic fissure) than the direct result of an eye growth defect.

Informed consent: Informed consent was obtained from all relevant subjects included in this paper. Patients shown in this review article were recruited as part of a national 'Genetics of Eye and Brain anomalies' study, approved by the Cambridge East Ethics Committee (04/Q0104/129). Additional informed consent for all individuals for whom identifying information is included in this article.

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FIGURE LEGENDS

Fig. 1 a. Facial view of a female with bilateral anophthalmia, carrying a *de novo* 14q22.3q23.1 deletion (chr14:57166658-5878654, hg19), including the gene *OTX2*. **b.** Facial view of a female diagnosed with bilateral anophthalmia, left cyst and hypoplastic optic chiasm, carrying a homozygous *ALDH1A3* pathogenic variant (Fares-Taie et al. 2013)

1.a.

1.b.

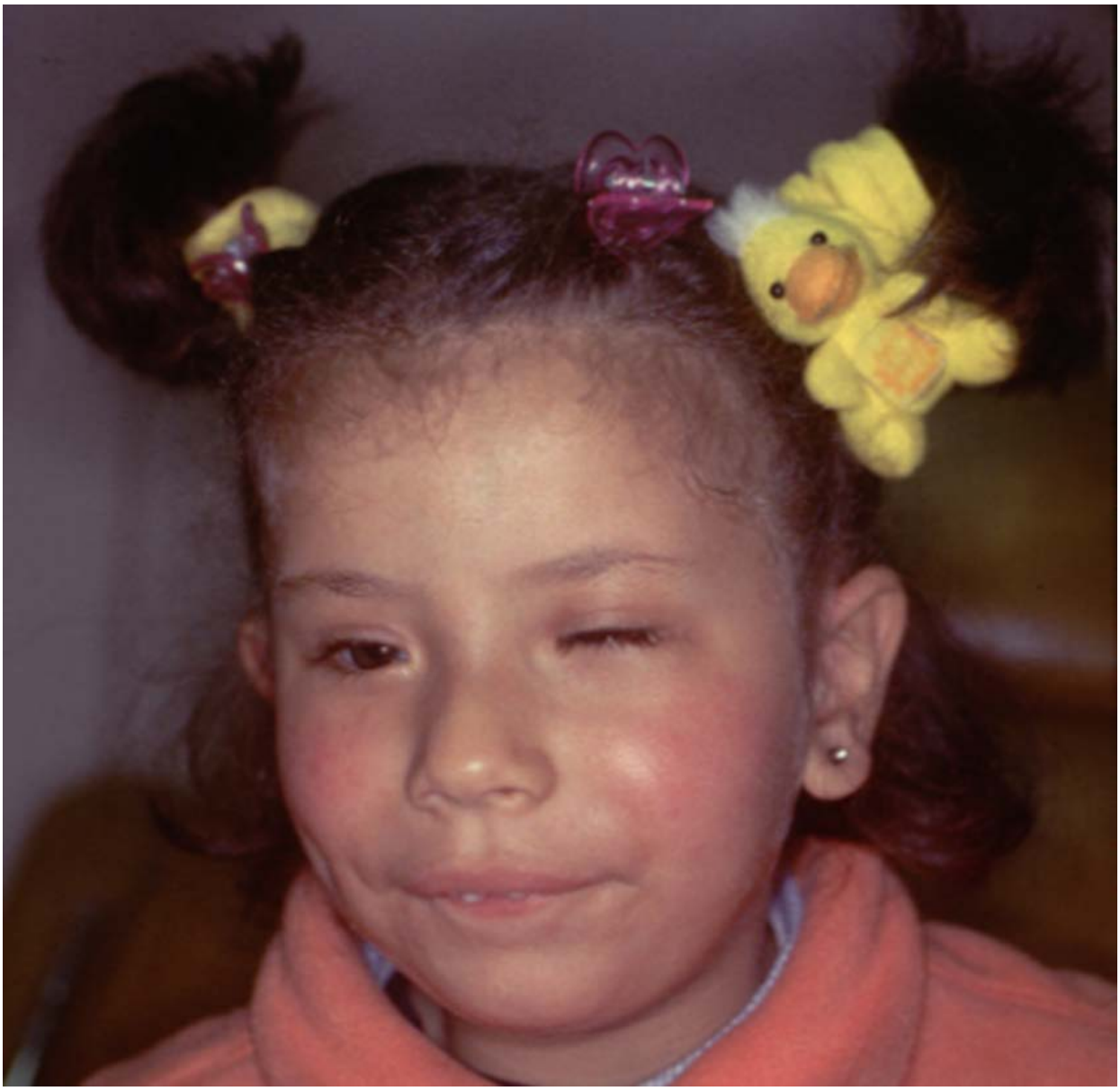
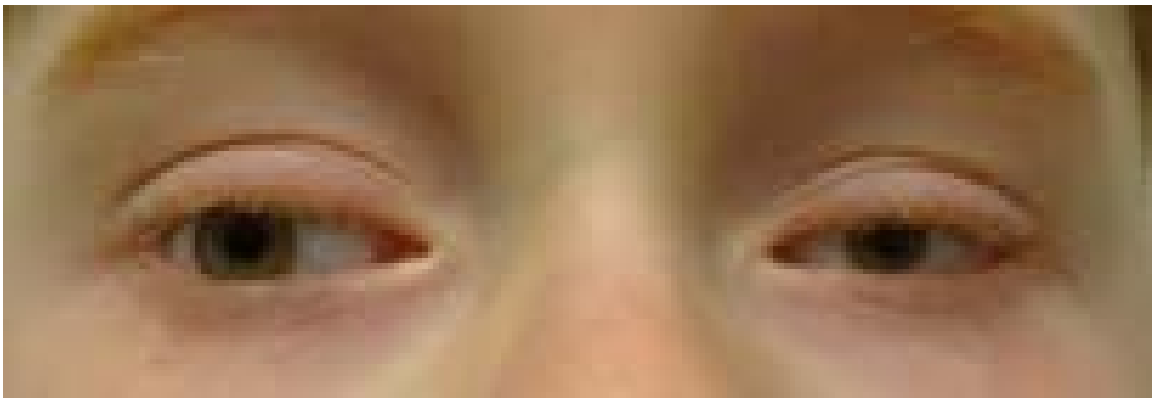


Fig. 2 a. Eyes of a male with non-syndromic bilateral colobomatous microphthalmia, with an inherited *YAP1* frameshift mutation (Holt et al. 2017). **b-c.** Magnified view of the eyes of the same individual. The right eye (b) presents with microphthalmia with chorioretinal coloboma involving optic disc. The left eye (c) also presents with microphthalmia with chorioretinal coloboma involving optic disc, more marked than right, and small convergent squint

2.a.



2.b-c.

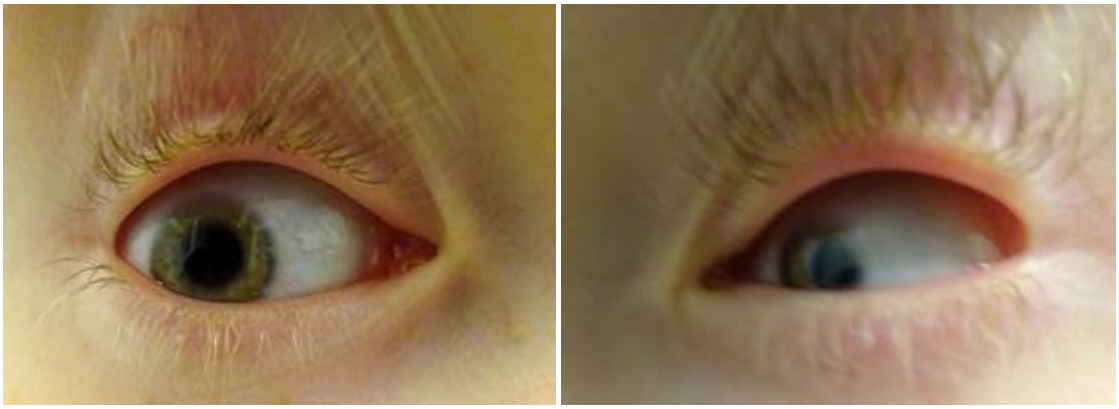
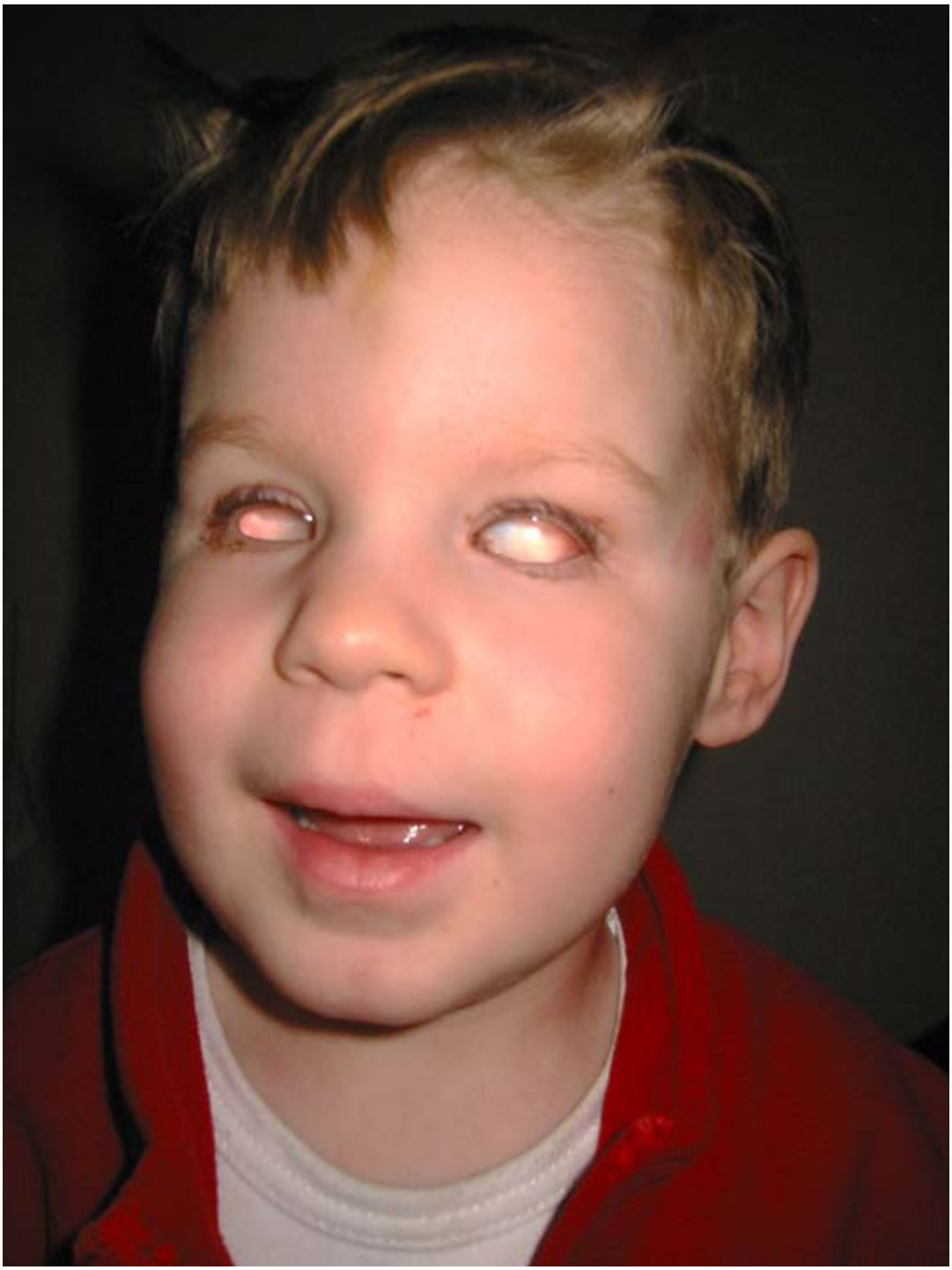


Fig. 3 a. Facial view of a male with clinical anophthalmia, carrying a *de novo PAX6* missense mutation. The patient is wearing bilateral clear prostheses as he has light perception, presumably from subconjunctival microphthalmic remnants. **b-c**. Right and left eyes, respectively, of a male diagnosed with bilateral microphthalmia, iris and optic disc coloboma, corneal opacity, bilateral congenital aphakia and primary glaucoma. The patient carries a missense variant in *GJA8* (Ceroni et al. 2018)

3.a.



3.b-c.