Clinical, genetic and biochemical signatures of *RBP4*-related ocular malformations

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Abstract

Background: The retinoic acid (RA) pathway plays a crucial role in both eye morphogenesis and the visual cycle. Individuals with mono- and bi-allelic pathogenic variants in *RBP4*, encoding a serum retinol specific transporter, display variable ocular phenotypes. Although few families have been reported worldwide, recessive inherited variants appear to be associated with retinal degeneration, while individuals with dominantly inherited variants manifest ocular development anomalies, mainly microphthalmia, anophthalmia and coloboma (MAC).

Method: We report here 7 new families (13 patients) with isolated and syndromic MAC harbouring heterozygous *RBP4* variants, to whom we performed biochemical analyses.

Results: For the first time, malformations that overlap the clinical spectrum of vitamin A deficiency are reported, providing a link with other RA disorders. Our data support two distinct phenotypes depending on the nature and mode of inheritance of the variants: dominantly inherited, almost exclusively missense, associated with ocular malformations, in contrast to recessive, mainly truncating, associated with retinal degeneration. Moreover, we also confirm the skewed inheritance and impact of maternal *RBP4* genotypes on phenotypic expression in dominant forms suggesting that maternal *RBP4* genetic status and content of diet during pregnancy may modify MAC occurrence and severity. Furthermore, we demonstrate that RBP blood dosage in patients could provide a biological signature crucial for classifying *RBP4* variants. Finally, we propose a novel hypothesis to explain the mechanisms underlying the observed genotype-phenotype correlations in *RBP4* mutational spectrum.

Conclusion: Dominant missense variants in *RBP4* are associated with MAC of incomplete penetrance with maternal inheritance through a likely dominant-negative mechanism.

KEYWORDS

Inheritance Patterns, Human Genetics, Ophthalmology, Eye Diseases, Genetics, Medical

Key messages

- **What is already known on this topic**

  Variants in *Retinol Binding Protein 4* (*RBP4)* have been associated with two ocular phenotypes: a recessively inherited retinal degeneration occasionally with coloboma, and a dominant form of microphthalmia, anophthalmia and coloboma. However, to date only few cases have been described and our knowledge about this gene is limited.

- **What this study adds**

  We described here the largest series of patients with dominant *RBP4*-related ocular malformations, expanded the clinical spectrum associated with variations in this gene and identified a common RBP blood profile among carriers.
  We also confirmed the skewed inheritance pattern of the dominantly inherited forms and proposed a novel theory to explain these correlations and the maternal transmission ratio distortion.

- **How this study might affect research, practice or policy**

  This study links for the first time the dominantly inherited *RBP4*-related phenotype to that observed in other retinoic acid pathway defects. The identified biochemical signature would help to classify variants detected in this gene. Thus, this work will be a useful resource both for clinicians by expanding the *RBP4*-related ocular and extra-ocular phenotype and for biologists by helping in establishing genetic diagnosis. Beyond this diagnostic consideration, these data open new avenues for deciphering molecular mechanisms involved in human diseases associated with defects in vitamin A homeostasis.

1 INTRODUCTION

Eye morphogenesis is a complex process requiring the close interplay of many genetic networks and signalling pathways to shape a three-dimensional ocular structure from the 4th to the 8th week of human gestation. Among them, the vitamin A (VA) pathway is essential both for proper development of eye structures and for the maintenance of vision throughout life with a crucial role in the visual cycle [1]. This pathway activity is dependent on dietary VA intake and the integrity of its components. It is well established that either an excess or deficiency of maternal retinoic acid (RA) results in multiple organ malformations in the fetus [2]. Besides the teratogenic effect of synthetic retinoids in the mother, several genetic defects affecting the transport or the metabolization of VA derivatives have been implicated in congenital eye malformation disorders, emphasising the importance of precise regulation of VA dosage during ocular development. The emblematic genetic disorder is one involving pathogenic variants in *STRA6* (*Stimulated by Retinoic Acid 6*), which encodes a cellular receptor that mediates the retinol intake into targeted cells. Many organs (including the eye) have been shown to express STRA6 receptors at their cell surfaces. Biallelic pathogenic variants in *STRA6* are involved in the characteristic PDAC (Pulmonary hypoplasia, Diaphragmatic anomalies, Anophthalmia/microphthalmia and Cardiac defects) spectrum (also named Matthew-Wood syndrome)[3] along with other RA signalling genes such as *RARB* [4], or more recently with *WNT7B* [5].

Mobilization of stored RA from the liver to the peripheral tissues is performed by transporters belonging to the Retinol-Binding Protein (RBP) family. The RBP4 protein is a monomeric-binding protein of 201 amino acids (aa) that, after cleavage of an 18 aa peptide signal, specifically transports vitamin A in plasma. In blood, the RBP4 protein is itself bound to prealbumin (also called transthyretin (TTR)). There are two forms of RBP: holo-RBP bound to retinol and apo-RBP free of retinol. Although dietary VA intake may vary, RBP4 and retinol concentrations are usually maintained within a narrow range under physiological conditions [1]. Alternative pathways are also able, at least partially, to maintain retinoid delivery to target cells, especially when *RBP4* function is disturbed [1,6].

Several pathogenic variants in *RBP4* have been implicated in both recessive and dominant phenotypes. Biallelic pathogenic variants of different types manifest mainly as retinal dystrophy, frequently associated with coloboma and acne [7–12]. Besides these biallelic *RBP4* mutational reports, there have also been a few descriptions of individuals carrying heterozygous *RBP4* missense

variants with a dominant inheritance pattern [13–16]. Heterozygous individuals display ocular malformations belonging to the microphthalmia, anophthalmia and coloboma (MAC) spectrum, with incomplete penetrance. Apart from one individual with neurodevelopmental delay [14], ocular involvement appears isolated in these dominant forms. Moreover, a parent-of-origin effect of the *RBP4* mutation is hypothesised because maternal transmission is significantly greater than paternal. It is thus suggested that when the *RBP4* mutation is transmitted by the mother, the VA delivery is decreased both at the placenta (involving maternal-derived RBP4) and later at the developing eye primordia (involving the fetal-derived RBP4). Thus, pathogenic *RBP4* variants are responsible for variable ocular phenotypes, the nature of which appears to depend on the type of variant and their inheritance patterns. In dominant forms, the occurrence of the phenotype appears to depend on the transmitting parent (present if inherited from the mother) and on the VA content of the diet during pregnancy [14].

In this report, we describe 13 new individuals displaying severe congenital ocular anomalies and variable extra-ocular features, from 7 families harbouring heterozygous (likely) pathogenic *RBP4* variants. These variants were exclusively missense and transmitted by the mothers.

2 MATERIAL AND METHODS

We collated clinical and genetic data from i) previously reported individuals with *RBP4* variants using the *PubMed* database and ii) patients referred for a genetic diagnosis of ocular malformations in our laboratories: the genetic laboratory of Toulouse University Hospital (TUH) (11 individuals) and Oxford Brookes University (2 individuals). The proband from the UK study (Family 3) was analysed by using whole genome sequencing (WGS). For all the other families, the genetic variant was identified using a customized 119 genes panel, including *RBP4* (NM_006744.4) (detailed in previous publications [13,17]) allowing both the detection of SNVs and CNVs in genes associated with isolated or syndromic micro-anophthalmia, anterior segment dysgenesis (ASD), aniridia, coloboma and congenital cataract. Variants were classified according to the ACMG guidelines [18]. Only those identified as ‘pathogenic’ or ‘likely pathogenic’ are reported here. Each variant was validated by Sanger sequencing. Blood quantification of retinol and RBP as well as TTR, kidney and hepatic profiles were performed in

specialised biochemistry laboratories (using either turbidimetric immunoassay or immunonephelometric method for the RBP dosage) on the available members of the 7 families. Novel variants described in this study have been submitted to the ClinVar database (www.ncbi.nlm.nih.gov/clinvar/).

3 RESULTS

a. Literature review

Recessive syndromic retinal dystrophies

Biallelic *RBP4* variants have been described in six families [7–12]. Their genotypes and phenotypes are summarized in Table 1. All affected individuals displayed retinal degeneration. Ocular malformations were noted in three families [9,11,12]. A majority of affected individuals displayed skin lesions consistent with acne. Variants were inherited from heterozygous asymptomatic parents. With the exception of two sisters described by Seeliger et al. [12] who had compound heterozygous missense variants, all variants were truncating variants (splice, nonsense or frameshift).

Table 1: Genetic, clinical and familial data of the *RBP4* recessive forms described in the literature. NA: not available.

Report	Sex	Genotype (NM_006744.4)	Ocular phenotype	Extraocular features	Family history and segregation
Cehajic-Kapetanovic et al., 2020	M	c.[67C>T];[67C>T] p.[(Arg23*)];[(Arg23*)]	Retinitis pigmentosa	Severe childhood acne vulgaris	Similar features in homozygous brother
Cukras et al., 2012	M	c.[111+1G>A];[111+1G>A] p.[?];[?]	Retinal degeneration, bilateral microcornea, cataract, iris and chorioretinal coloboma	Patent ductus arteriosus	Heterozygous asymptomatic parents Consanguinity Homozygous sister (55y) with retinal dystrophy and severe acne
Kessel et al., 2022	F	c.[112-2A>G];[112-2A>G] p.[?];[?]	Early onset atypical retinitis pigmentosa	Rheumatoid arthritis acne	Similar phenotype in homozygous brother Bilateral microcornea, sclerocornea, corectopia and optic nerve malformations in her heterozygous daughter
Seeliger et al., 1999	F	c.[176T>A];[278G>A] p.[(Ile59Asn)];[(Gly93Asp)]	Retinal degeneration, subtle bilateral iris coloboma	Severe comedogenic acne and widespread follicular keratosis	Similar features in the compound heterozygous sister Heterozygous asymptomatic mother carrying p.(Ile59Asn) variant
Khan et al., 2017	F	c.[248+1G>A];[248+1G>A] p.[?];[?]	Retinal degeneration, bilateral iris and retinal coloboma, unilateral microphthalmia	Acne-like skin manifestations	Consanguineous pedigree
Colombo et al., 2021	M	c.[457_459delTTC];[457_459delTTC] p.[(Phe153del)];[(Phe153del)]	Retinitis pigmentosa	None	Heterozygous healthy mother No data available on family.

Dominant eye malformations

Only a few dominant forms of *RBP4*-related eye disease have been reported to date in the literature. Their genotypes and phenotypes, as well as those of our cohort, are summarized in Table 2. Chou et al. reported the p.(Ala75Thr) variant in a large family with several affected individuals, whose phenotypes ranged from asymptomatic to MAC spectrum malformations of various severity [14]. The pedigree suggested autosomal dominance with incomplete penetrance. In parallel, they reported the p.(Ala73Thr) variant in two unrelated individuals (a male with bilateral anophthalmia and neurodevelopmental delay and a female with unilateral colobomatous microphthalmia), both with maternal transmission of the variant. These two missense variations were hypothesized to have a dominant-negative effect and to compete with the wild-type protein for STRA6 occupancy. Interestingly, Kaur et al. also reported two siblings (fetuses) with isolated bilateral anophthalmia carrying the same p.(Ala73Thr) variant transmitted by the asymptomatic mother [15]. Riera et al. reported the p.(Tyr132Asn) variant in an individual with isolated bilateral complex microphthalmia [16]. Finally, a heterozygous *RBP4* splicing variant (c.569-1G>A p.?) was identified in 75-year-old man with isolated and sporadic bilateral retinal coloboma [13]. For the latter two variants, parental segregation was unavailable.

Table 2: Genetic, clinical and familial data of the *RBP4* dominant forms described in the literature and in this study. NA: not available. WA: Weeks of amenorrhea.

Report	Sex	Genotype	Ocular phenotype	Extraocular features	Family history and segregation
Chou C et al., 2015	M	c.217G>A p.(Ala73Thr)	Bilateral anophthalmia	Neurodevelopmental delay	Inherited from asymptomatic mother
Chou C et al., 2015	F	c.217G>A p.(Ala73Thr)	Unilateral microphthalmia and coloboma		Inherited from asymptomatic mother
Kaur A et al., 2022	NA	c.217G>A p.(Ala73Thr)	Interrupted pregnancy (20 WA) for isolated bilateral anophthalmia		Inherited from asymptomatic mother Second interrupted pregnancy for isolated bilateral anophthalmia.
This study Family 1	M	c.217G>A p.(Ala73Thr)	Bilateral microphthalmia with retinal dysplasia	Right lung lobulation delay and brachymesophalangia of the fifth fingers	Inherited from asymptomatic mother Second interrupted pregnancy (19 WA) for bilateral complex microphthalmia and delayed right pulmonary segmentation with only two formed lobes.
This study Family 2	F	c.218C>T p.(Ala73Val)	Bilateral anophthalmia	Intellectual deficiency autistic features	Inherited from asymptomatic mother Isolated unilateral iris coloboma and bilateral severe myopia in her brother
Chou C et al., 2015	11 affected members	c.223G>A p.(Ala75Thr)	4 with anophthalmia 7 with microphthalmia 9 obligate carriers	1 with atrial septal defect 1 with cerebral aneurysm	Inherited from asymptomatic mother in 10 out of the 11 patients
This study Family 3 (434)	F	c.271A>G p.(Met91Val)	Bilateral severe microphthalmia/ anophthalmia	Speech delay, atrophy of the cerebral cortex, delayed brain myelination, corpus callosal hypoplasia, and hippocampal anomaly	Inherited from the asymptomatic mother and carried by the affected maternal grandmother
This study Family 4	M	c.358G>T p.(Asp120Tyr)	Bilateral Microphthalmia with colobomateous cysts and microcornea	Intra-uterine growth retardation	Recurrence of bilateral microphthalmia in twin fetuses, inherited from asymptomatic mother

Report	Sex	Genotype	Ocular phenotype	Extraocular features	Family history and segregation
This study Family 5	F	c.358G>C p.(Asp120His)	Unilateral iris and retinal coloboma		Parental segregation analysis unavailable
This study Family 6	F	c.383A>G p.(Asp128Gly)	Bilateral anterior segment dysgenesis		Maternal transmission. Symptomatic mother (early cataract with bilateral posterior embryotoxon) Maternal grand-parents deceased.
Riera M et al., 2017	M	c.394T>A p.(Tyr132Asn)	Bilateral complex microphthalmia		Segregation analysis was not done
This study Family 7	F	c.394T>A p.(Tyr132Asn)	Unilateral colobomatous microphthalmia	Hypotonia, delayed motor skills, interventricular communication, dolichocolon, short stature	Inherited from the asymptomatic mother
Aubert-Mucca M et al., 2021	M	c.569-1G>A p.?	Bilateral retinal coloboma		Segregation analysis was not done

b. This report

In this study, we report 7 new families with heterozygous *RBP4* (likely) pathogenic variants (classification details are available in Supplementary Table 1). Their genotypes, phenotypes and family histories are detailed below, and summarized in Table 2 and Figure 1.

Family 1

The proband is a male fetus terminated at 24 weeks' gestation following a diagnosis of severe bilateral microphthalmia on ultrasound. Autopsy findings identified additional features including retinal dysplasia, right lung lobulation delay and brachymesophalangy of both fifth fingers. Genetic testing was initiated after a second terminated pregnancy at 19 weeks' gestation of a female fetus with bilateral complex microphthalmia. Autopsy showed delayed right pulmonary segmentation with only two formed lobes. Ocular histology revealed bilateral microphthalmia with thickened sclera and cornea, absence of the lens, rudimentary choroid and retinal dysplasia with rosette-like structures. For both foetuses, array-CGH was normal, while targeted NGS revealed heterozygous, pathogenic *RBP4* variant c.217G>A p.(Ala73Thr), inherited from their asymptomatic mother (Supplementary Table 1). This variant has previously been associated with MAC spectrum malformations [14,15].

Family 2

The proband is a young girl with syndromic bilateral anophthalmia. She was born at term with normal growth parameters. She has neurodevelopmental delay (walked at 4 years-old, says only few words) and developed sleeping disturbances, autistic features with difficulties in social interaction, and some aggressive behaviour. Cardiac and renal ultrasounds were normal. Cerebral MRI revealed bilateral optic nerve and chiasmal hypoplasia. She is the youngest of four siblings, born from unrelated healthy parents. Her elder brother displayed unilateral iris coloboma and bilateral severe myopia. Of note, the first-born female child died after few days of life following neonatal infection and cerebral haemorrhage, having been born prematurely (28 weeks' gestation).

Array-CGH performed on the proband was normal. Targeted NGS analysis identified a heterozygous *RBP4* variant c.218C>T p.(Ala73Val), considered likely pathogenic (Supplementary Table 1). A

pathogenic variant affecting the same amino acid (p.(Ala73Thr)) has been reported in two unrelated families [14,15]. Segregation analysis demonstrated the variant was inherited from the asymptomatic mother and also transmitted to the proband's symptomatic brother. The mother reports no difference in diet between the pregnancies. WGS in the proband did not identify any other molecular explanation for her severe neurocognitive phenotype. Measurement of RBP in plasma showed low levels in the proband, her mother and her brother (Figure 1 and Supplementary Table 2). Retinol was also reduced below the normal range in the proband and her mother, and in the inferior normal range in the brother (Figure 1 and Supplementary Table 2). TTR, hepatic and renal biochemical profiles were normal in each individual.

Family 3

The proband is a young girl conceived through *in vitro* fertilisation from unaffected parents. The pregnancy was uneventful, except for the mother being treated with heparin for thrombophilia until a scan at 30 weeks' gestation revealed intrauterine growth retardation. An elective Caesarian section was performed at 36 weeks: birth weight 2.2kg (16e percentile), length 44cm (12e percentile), head circumference 33cm (58e percentile). Bilateral 'anophthalmia' was diagnosed on day 4. Assessment at 12 weeks revealed possible light perception on the right, ocular ultrasound revealed bilateral microphthalmia: axial length was 7.8mm with a thin lens *in situ* in right eye and 4.7mm with a total retinal detachment in left eye. At age 11 weeks, she had a weight of 4.2kg (-2.6SD), head circumference of 35.6cm (-4.5SD) and length of 56.5cm (-1.6SD). Early cardiac and renal ultrasound were normal. Cranial MRI revealed some atrophy of the cerebral cortex, delayed brain myelination, corpus callosum hypoplasia and hippocampal anomaly. At 10 months-of-age, her weight was 7kg (-1.6SD), normal length and head circumference 41cm (-2.5SD). Her development has been normal considering her visual impairment, except for some speech delay. The mother's ocular examination was normal, apart from left optic disc drusen. The maternal grandmother had congenital right microphthalmia, with microcornea and a small cyst; the left anterior segment was normal, with age-related nuclear sclerosis (cataract), obscuring a clear view of the fundus, and which showed a tilted anomalous optic disc.

WGS analysis revealed a heterozygous likely pathogenic c.271A>G p.(Met91Val) variant in *RBP4* in the proband, unaffected mother and affected maternal grandmother (Supplementary Table 1). Array-CGH revealed a paternally inherited duplication at Xp22.13(17700437_17782056)x3pat. The region of imbalance contains two protein coding genes; a whole gene duplication of *SCML1* and a partial duplication (exons 2-8) of *NHS*. Loss-of-function variants in *NHS* are associated with X-linked cataracts and Nance Horan syndrome, a dominant X-linked condition characterized by congenital cataract, dental anomalies, dysmorphic features and in some cases intellectual disability. The effect, if any, of a partial duplication of this gene is unknown.

Family 4

The proband is a male foetus terminated at 26 weeks' gestation after an ultrasound diagnosis of intra-uterine growth retardation and bilateral microphthalmia. Ocular histological examination confirmed bilateral microphthalmia with colobomatous cysts and microcornea (2mm in diameter). The parents were unrelated and healthy. Their second pregnancy produced a healthy boy at term. During their third pregnancy, an ultrasound at 19 weeks' gestation confirmed male twins (monochorionic biamniotic) both of whom had bilateral microphthalmia, and the parents elected to terminate the pregnancy. Karyotype and array-CGH were normal in the fetuses. A heterozygous *RBP4* variant c.358G>T p.(Asp120Tyr)**,** classified as likely pathogenic, was detected in the first fetus, two symptomatic twin fetuses as well as the asymptomatic mother (Supplementary Table 1). Measurement of RBP and retinol in the mother show low levels in blood.

Patient 5

The proband is a young female, born to unrelated parents. She presented with isolated unilateral left iris and retinal coloboma. Echocardiography and renal ultrasound were normal. Panel sequencing revealed the heterozygous likely pathogenic c.358G>C p.(Asp120His) variant in *RBP4* (Supplementary Table 1). Unfortunately, samples from her asymptomatic parents were not available for analysis. The RBP plasma and the retinol levels in the proband were below the normal range (Supplementary Table 2). TTR, hepatic and renal biochemical profiles were normal.

Family 6

The proband is a young girl presenting with isolated bilateral ASD (right Axenfeld-Rieger anomaly and left posterior embryotoxon). Her mother developed bilateral cataract at 25 years of age and ophthalmological examination revealed associated bilateral posterior embryotoxon. There was an history of severe myopia in several maternal siblings. Both the proband and her three asymptomatic brothers carry the maternally inherited c.383A>G p.(Asp128Gly) *RBP4* variant, classified as likely pathogenic (Supplementary Table 1). Measurement of RBP in plasma show low levels in all five heterozygous individuals in contrast with the father not carrying the variant (Figure 1). Retinol was reduced in the proband and two of her asymptomatic brothers, but was in the inferior normal range in one of her asymptomatic brothers and the mother (Supplementary data 2). Of note retinol was the in upper normal range in the non-mutated father.

Family 7

The proband is a young girl, the first of two siblings born to unrelated healthy parents. At birth, unilateral colobomatous microphthalmia was noticed, as well as a heart murmur corresponding to a small ventricular septal defect that closed spontaneously. She had early motor delay (hypotonia, walking at 27 months) with subsequent resolution (normal walking and running). Her cognitive skills have always been within the normal range. She received surgery for a dolichocolon and pathological examination excluded a diagnosis of Hirschsprung disease. She is also followed for a short stature (-2SD for the size and weight) and head circumference (+0.5SD). Array-CGH (100K) was normal, while panel sequencing identified a heterozygous likely pathogenic c.394T>A p.(Tyr132Asn) variant in *RBP4* (Supplementary Table 1). This variant has been reported once previously associated with a colobomatous microphthalmia phenotype [16]. Segregation analysis showed the presence of the variant in the asymptomatic mother. The RBP plasma level as well as the retinol dosage in the proband were below the normal range (Supplementary data 2) with otherwise normal TTR, hepatic and renal markers.

Table 3: Details of missense variants reported to date and their structural location in the mature RBP4 protein

RBP4 is stabilized by three disulphide bonds linking the following residues: 4-160, 70-174 and 120-130 on the mature protein. In bold, missense variations found in compound heterozygosity (Seeliger MW et al, 1999). LOF = Loss-Of-Function. DN = Dominant Negative (Chou C et al 2015). *In contact with the retinol (Cowan 1990, Zanotti G, 2004). **Salt link with the residue R121 (Zanotti G, 2004).

Missense pathogenic variants	Residue position before N-terminal cleavage of the 18 aa peptide signal	Residue position in the mature protein (after cleavage)	Structural location on the mature RBP4 protein	*In vitro* demonstrated mechanism
p.Ile59Asn	59	41	β strand B	LOF
p.Ala73Thr	73	55*	β strand C	DN
p.Ala75Thr	75	57*	β strand C	DN
p.Met91Val	91	73*	β strand D	
p.Gly93Asp	93	75	β strand D	LOF
p.Asp120His	120	102**	β strand F	
p.Asp120Tyr	120	102**	β strand F	
p.Asp128Gly	128	110	Hairpin F-G	
p.Tyr132Asn	132	114	β strand G	

4 DISCUSSION

Ocular phenotype in dominant *RBP4* forms: MAC spectrum

Until now, only a few individuals with dominantly inherited *RBP4* variants and ocular malformations have been described in the literature (6 families in total). Through our report of 13 new individuals, we provide additional evidence for the role of *RBP4* in dominantly inherited ocular disorders with incomplete penetrance. In the majority of cases, the ocular phenotype consists of bilateral and severe eye anomalies belonging to the MAC spectrum. Intriguingly, the phenotype of one patient is out of this MAC spectrum, who displays isolated anterior segment dysgenesis (the proband of family 6) and carries the p.(Asp128Gly) variant inherited from his mother, herself presenting with early onset cataract.

Although one fetus showed bilateral microphthalmia and retinal dysplasia (family 1), there was no retinal degeneration noted in patients with heterozygous disease-causing variants.

Extra-ocular phenotype in dominant *RBP4* forms: non-specific RA signaling features

Most individuals with dominantly inherited *RBP4* pathogenic variants display isolated ocular malformation (22/30 patients). Some exhibit extra-ocular features (8/30 patients) consisting mainly of neurodevelopmental disorders, but also brain malformations, lung lobulation anomalies, growth retardation, cardiac defects and dolichocolon. Although belonging to the RA pathway, the *RBP4*-related phenotype only partly overlaps the PDAC syndrome observed in individuals with variants in other genes of this pathway. Nevertheless, most of the extra-ocular signs observed in *RBP4*-mutated individuals can be included in this RA clinical spectrum. It is likely that other pathways may partly compensate RBP-mediated VA transport to most organs except for the eyes, which are highly dependent on VA both for development and vision [6]. As some recurrent variants were associated with both isolated ocular and syndromic forms (p.(Ala73Thr) and p.(Asp120Tyr)), there does not seem to be a clear explanation for the severity of systemic involvement, and other factors such as maternal diet may have affected fetal VA levels during pregnancy.

Genotype-phenotype correlations

Recessive versus dominant phenotypes

From this review, two phenotypes are emerging. Firstly, a recessive phenotype consisting of a retinopathy with an additional, quite frequent, ocular coloboma, and acneiform skin lesions (Table 1). Secondly, a dominant phenotype including MAC and systemic features with incomplete penetrance and parent-of-origin effect (Table 2) (discussed below). These two phenotypic spectra are depending on the *RBP4* genotype. Such correlations between genotype and inheritance pattern and between inheritance pattern and presentation of the disease are described in various other genetic diseases [19–22].

The recessive phenotype is observed in patients carrying biallelic putative loss-of-function alleles of different types (missense, splice and nonsense variants, as well as in-frame deletions) (Figure 2A). Of note, the heterozygous daughter of the female proband in the recessive family reported by Kessel et al. [10] displayed bilateral eye malformation evocative of the dominant phenotype. This is the only reported case manifesting ocular signs among the heterozygous carriers of recessive forms. Nevertheless, another genetic cause was not excluded in this atypical member of the family.

Moreover, other carrier relatives in this and other "recessive" families are asymptomatic, unsupportive of the effect of these variants in the heterozygous state [9]. In this family, it is possible that the suspected complete absence of functional RBP4 protein due to the homozygous variation in the mother may lead to a decrease in the transplacental retinol exchange, contributing to the ocular developmental defect in the heterozygous daughter. However, there is no similar case in the literature to support this hypothesis. It may reflect the possible impact of maternal dietary VA deficiency during pregnancy.

In contrast, the dominant phenotype is observed in patients carrying almost exclusively missense RBP4 variants (all except one) (Table 2).

Position and structural location of the missense mutations

After cleavage of the signal peptide (18 aa), the mature structure of the RBP4 protein consists of a single polypeptide chain containing 183 amino acids, stabilized by three disulphide bridges and including an N-terminal coil, eight antiparallel β-strands (A–H) and a short α-helix close to the C-terminus [23]. The β-barrel core is able to specifically host one molecule of retinol, allowing this hydrophobic vitamin to be transported through the bloodstream. To date, all the missense mutations described in *RBP4* (regardless of the mode of transmission) are located in the β-barrel core of the protein (Figure 2B, Table 3) [24,25]. Each of these missense variants is located in one of the eight β-strands, with the exception of the p.(Asp128Gly) variant located in the hairpin F-G of the β-barrel (Figure 2B, Table 3). Intriguingly, this p.(Asp128Gly) variant is carried by the only patient presenting with an isolated ASD (i.e without MAC). With the exception of this missense variant associated with an atypical phenotype (isolated ASD), there does not seem to be a correlation between the missense position and inheritance pattern (see below).

Different molecular mechanisms related to the missense mutations

To try to explain the difference in inheritance patterns observed among the different missense variants, Chou et al. [14] studied the two compound heterozygous missense variants (p.(Ile59Asn) and p.(Gly93Asp)) [12] and demonstrated a loss-of-function effect for both as a result of protein misfolding and increased cellular retention (Table 3). In contrast, functional studies performed on the

two “dominant” missense variations (p.(Ala73Thr) and p.(Ala75Thr)) have suggested a dominant-negative effect of the mutant protein [14]. Indeed, the dominant-negative RBP mutant protein was found to bind retinol poorly but to occupy the STRA6 receptor with much higher affinity than the wild-type RBP protein. Therefore, they suggested a difference in mechanisms amongst mutations that could explain difference in inheritance pattern.

Of note, the same hypothesis of dominant-negative mechanism can be formulated for the splicing variant identified by Aubert-Mucca et al [13]. This variant affects the canonical site of the last exon c.569-1G>A (Figure 2A) and, consequently, is unlikely to be subject to Nonsense-Mediated Decay (NMD). Instead, it is predicted to abolish the canonical acceptor site and to create a new one, which includes the first nucleotide of the last exon, causing a frameshift resulting in a modification of the 12 last amino acids of the protein followed by a 13 amino acids protein extension (p.(Gly190Valfs*25)). Such a modification of the C-terminal part of the protein may be responsible for a dominant-negative effect as described for other genes [19,21].

However, it still remains unclear how monoallelic pathogenic variations with a putative dominant-negative effect may lead to a severe developmental phenotype compared to the biallelic loss-of-function ones.

Impact on retinol bidirectional cellular transport?

Other delivery pathways are known to bring retinol to cells [1]. In particular, receptor-independent diffusions of retinol through cell membrane may occur, especially upon RBP4 receptor dysfunction [26]. Thus, even in the absence of RBP, the retinol homeostasis could be maintained in the cells by compensatory pathways and the intracellular concentration may be sufficient for proper ocular development in recessive families.

According to the works of Chou et al. the dominant-negative RBP mutant protein is binding retinol poorly and binds the STRA6 receptor with more affinity than the holo-RBP [14]. Several studies [27–29] have shown that STRA6 is able to transport retinol bidirectionally and that the activity of STRA6 in vitamin A influx or efflux is depending on its extracellular interaction with holo-RBP or apo-RBP. In particular, they showed that the binding of apo-RBP promotes retinol export out of the cells. Thus, if we consider that the mutant RBP (equivalent to apo-RBP given its decreased affinity to retinol) is

bound to STRA6 with more affinity than holo-RBP [14], one can imagine than the mutant RBP will promote retinol efflux and dramatically deplete the cell in retinol, worsening the VA deficiency and leading to ocular developmental defects. Knowing that organs, such as the eye, whose retinol content during development is highly dependent on the presence of STRA6, whose expression is also high at certain stages of embryogenesis [30], it could be a plausible explanation of the severe eye defects observed in dominant families. The same would be observed at the level of placenta (explaining the maternal bias in inheritance) and the organs expressing high level of STRA6 during embryogenesis (explaining the PDAC spectrum manifestations in those dominant forms).

Biochemical signature

Chou et al. showed that 3 members of the family carrying the heterozygous variant p.(Ala75Thr) had normal circulating RBP, but reduced VA levels in serum [14]. Unfortunately, they did not biochemically test the two probands carrying the p.(Ala73Thr). In literature, the few homozygous individuals with recessive variants who have been tested displayed very low or undetectable retinol and RBP levels [10,12] and the two heterozygous unaffected carriers of two unrelated “recessive” families have normal retinol levels but, again, reduced RBP in blood [9,12]. Thus, these blood dosages were consistent with the hypothesis of different mechanisms depending on the mutations [14].

In our study, blood quantification of retinol and RBP in *RBP4* heterozygotes (symptomatic and asymptomatic carriers) in 5 of the 7 dominant families revealed low levels of RBP in all tested individuals who had otherwise normal TTR, kidney and hepatic profiles. The retinol level was under the normal range in 8 out of 11 patients, and in the inferior normal range in the remaining three. In family 6, the father (not bearing the pathogenic variant) had retinol in the upper normal range, thus arguing for a direct effect of the pathogenic variation on retinol blood levels rather than an effect of the familial diet.

Although RBP dosages cannot be used to differentiate a “dominant” or a “recessive” mutation as both lead to a decreased in RBP levels in heterozygous carriers, our results suggest that a biochemical quantification of RBP could be an important adjunct for interpreting missense variants in *RBP4*. Decreased RBP blood levels in patients bearing *RBP4* missense variations could then be used as a

criterion of pathogenicity when classifying the variants (PP4 criterion from the ACMG classification [18]), while normal levels could not rule out the pathogenicity of the variant.

Maternal genetic status and content of vitamin A diet during pregnancy

Combination of maternal VA diet during pregnancy and genetic status seems to impact the occurrence and severity of the ocular phenotype observed in a *RBP4*-mutated fetus [14]. Vitamin A dependent organogenesis relies on both the ability of fetal RBP to transport retinol to the organs and maternal RBP to bring retinol to the placenta, the amount of retinol itself also depending on the VA diet of the mother during pregnancy. Thus, the probability of the fetus being severely affected is correlated with the genetic status of the fetus and mother, as well as maternal VA dietary content during pregnancy. With the exception of one family from literature [14], dominant forms were exclusively reported in individuals who had inherited heterozygous *RBP4* variants from an asymptomatic mother. Moreover, in the solely reported paternal transmission [14], the penetrance was incomplete (only one of the twins being affected) and the phenotype mild (unilateral coloboma). In this latter case, the twin pregnancy may explain lower blood retinol levels compared to single pregnancy, thus mimicking a decrease in retinol transplacental exchange observed when the variation is inherited from the mother. All three heterozygous mothers that we tested in this study (families 2, 4, 6) show a low level of RBP dosage that is compatible with the hypothesis that, when the *RBP4* variant is maternally inherited, the VA delivery (involving maternal-derived RBP4) is necessarily decreased at the placenta.

We also note a difference in severity between the two symptomatic children in family 2 (both bearing the p.(Ala73Val) variant), raising the question of the impact of maternal diet during pregnancy as previously proposed [14].

5 CONCLUSIVE REMARKS

Variants in *RBP4* are associated with two different phenotypes and inheritance patterns, depending on their nature. Recessive *RBP4* loss-of-function variants are associated with retinal degeneration. The retinopathy is thought to result from a degenerative process secondary to the prolonged absence

of the VA substrate to photoreceptors which impairs the visual cycle [9]. The partial or total absence of RBP4 is apparently only deleterious during adulthood and the presence of coloboma in some affected individuals suggests incomplete compensation of VA intake to the eye during development. This degenerative process contrasts with the malformative process observed for the putative dominant negative alterations [13]. In dominant forms, the congenital occurrence of ocular defects is probably due to greatly reduced VA delivery to the eye during embryogenesis. As we suggest in this work, it is possible that the presence of a dominant-negative mutant RBP impact the retinol cellular transport leading to an imbalance in retinol flux, depleting the cell in retinol and thus at the origin of a severe phenotype during embryonic development. The severity of those ocular phenotypes (ranging from ASD or coloboma to anophthalmia) would then be modulated both by the genetic status of the mother and VA diet during pregnancy. We confirm here that maternal transmission significantly increases the penetrance of *RBP4* deficiency (100% of our cases inherited from the mother). The VA deficiency is evidenced by quantification of low levels of RBP and retinol in serum of patients. Strikingly, *RBP4* heterozygous carriers of “dominant” mutations display the same biochemical profile (RBP reduced in blood) than those with heterozygous “recessive” mutations. The few homozygous individuals with recessive variants that have been tested displayed very low or undetectable retinol and RBP levels [10,12]. This biochemical signature would help to classify variants detected in this gene.

In the light of these novel data, further studies are required to fully understand the effect of *RBP4* pathogenic variants and their mechanisms in this very intriguing maternal inheritance pattern.

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COMPETING INTERESTS

There are no competing interests for any author.

ETHICS APPROVAL STATEMENT

TUH has pledged to uphold France's CNIL MR-004 standards, which govern the use of data for medical research not directly involving human subjects. The TUH Data Protection Officer found this study compliant with the EU General Data Protection Regulation. It was included in both the TUH retrospective study registry (as RNIPH #2021-44) and the CNIL MR-004 registry (as #2206723 v 0). TUH approved this research and acknowledges that it has met all ethical obligations. The UK cohort of 430 individuals with ocular developmental anomalies was recruited as part of a national 'Genetics of Eye and Brain Anomalies' study (REC 04/Q0104/129).

CONTRIBUTORSHIP STATEMENT

JP and NC did the study design. JP, BC, NC wrote the manuscript. JM, SW, DR, SAB, PM, SG, IP, DB, NR did patients' evaluation. JP, NC and RH did the genetic analysis. All the authors have reviewed and approved the manuscript.

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FIGURES LEGENDS

Figure 1: Pedigrees of the 7 families reported in this study. **RBP**: serum RBP dosage ; **Ret**: serum retinol dosage ; ↓: below normal range ; →: within normal range. Black symbols represent MAC spectrum while grey symbols represent ASD.

Figure 2: A. Schematic representation of *RBP4* containing 5 coding exons (solid blue). The variants represented above have been described in the recessive forms (in red, the truncating variants; in orange, the missense ones and in black the in-frame deletion). The variants represented below are those described in the dominant forms (in orange, the missense ones and in black the mutation with CTE). **B.** AlphaFold structure prediction of the RBP4 protein (https://alphafold.ebi.ac.uk/entry/P02753). Amino acids reported with a causal variant in the literature affect predominantly ß strands.