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THÈSE

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DIPLÔME D'ÉTAT DE DOCTEUR EN MÉDECINE

Qualification en GENETIQUE MEDICALE

DIAGNOSIS OF TUBEROUS SCLEROSIS IN THE PRENATAL PERIOD: A RETROSPECTIVE STUDY OF 240 CASES AND REVIEW OF THE LITERATURE

Diagnostic de la sclérose tubéreuse de Bourneville en période prénatale : une étude rétrospective de 240 cas et revue de la littérature

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Né le 01/08/1994 à Bernay (27)

Sous la direction de Mme COLIN Estelle

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Abbreviations list

ACMG / AMP	American College of Medical Genetics and Genomics / Association for Molecular Pathology
ClinGen SVI	Clinical Genome Sequence Variant Interpretation
CNS	Central Nervous System
CR	Cardiac Rhabdomyoma
CT	Cortical Tuber
DD	Definite Diagnosis
DHPLC	Denaturing High-Performance Liquid Chromatography
DNA	DesoxyriboNucleic Acid
EEG	ElectroEncephaloGraphy
FDIU	Fetal Death *In Utero*
Indel	Insertion-deletion
IUGR	IntraUterine Growth Restriction
LA	Left Atrium
LOVD	Leiden Open Variation Database
LV	Left Ventricle
MLPA	Multiplex Ligation-dependent Probe Amplification
MRI	Magnetic Resonance Imaging
mRNA	messenger RiboNucleic Acid
mTOR	mammalian Target Of Rapamycin
NGS	Next-Generation Sequencing
NVI	No Variant Identified
PCR	Polymerase Chain Reaction
P/LP	Pathogenic / Likely Pathogenic
RA	Right Atrium
RV	Right Ventricle
SEN	SubEpendymal Nodule
SNV	Single Nucleotide Variant
SV	Structural Variation
TOP	Termination Of Pregnancy
TOSCA	TuberOus SClerosis registry to increase disease Awareness
TSC	Tuberous Sclerosis Complex
US	UltraSound
VUS	Variant of Unknown Significance
WG	Weeks of Gestation

PLAN

DIAGNOSIS OF TUBEROUS SCLEROSIS IN THE PRENATAL PERIOD: A RETROSPECTIVE STUDY OF 240 CASES AND REVIEW OF THE LITERATURE

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ABSTRACT

Tuberous sclerosis complex (TSC) is a rare multisystemic disorder caused by a pathogenic variant in the *TSC1* or *TSC2* gene. A great phenotypic variability characterizes TSC. The condition predisposes to the formation of hamartomas in various tissues, neurologic and neurodevelopmental disorders such as epilepsy, psychiatric disorders, as well as intellectual disability in 50%. TSC may be responsible for cardiac rhabdomyomas (CRs), cortical tubers, or subependymal nodules during fetal life. Detecting multiple CRs is associated with a very high risk of TSC, but the CR could be unique and isolated. Few data exist to estimate the risk of TSC in these cases. We report the largest series of prenatal genetic tests for TSC with a retrospective study of 240 fetuses presenting with suggestive antenatal signs. We also provide a review of the literature to specify the probability of clinical or genetic diagnosis of TSC in case of detection of unique or multiple CRs. Indeed, an early diagnosis is crucial for the counseling of the couple and their families. In this series, a definite diagnosis was assessed in 50% (41/82) of fetuses who initially presented with a unique CR and 80.3% (127/158) in case of multiple CRs. We detected 3 cases of parental germinal mosaicism and estimated the risk at 2.6% (3/115).

INTRODUCTION

Tuberous sclerosis complex (TSC) is a genetic disorder first described in 1862 by von Recklinhausen. Bourneville provided a more complete characterization in 1880; hence, the condition is also called Bourneville's disease[1]. Causal variants involve either the *TSC1* gene (9q34, MIM 605284), coding for hamartin, or *TSC2* (16p13, MIM 191092), coding for tuberin. TSC is transmitted with an autosomal dominant inheritance. Hamartin and tuberin act together to form the TSC complex, a ubiquitous heterodimer with a GTPase activity carried by tuberin. The TSC complex regulates cell growth and proliferation by inhibiting the mammalian target of rapamycin (mTOR) signaling pathway. If harmatin or tuberin is abnormally produced due to the presence of a pathogenic variant in *TSC1* or *TSC2*, the TSC complex is inactivated, leading to an upregulation of mTOR. This mechanism is responsible for the formation of benign hamartomas in various tissues and sometimes more aggressive neoplasms[2,3].

TSC is a multi-system disorder with a prevalence estimated between 1/5 800 and 1/13 520 live births[4,5], characterized by great inter- and intra-familial variability in age of onset and severity. The updated 2021 diagnostic criteria require two major features or the association of one major and two minor features for a definite clinical diagnosis[6]. Major features include skin (hypomelanotic macules, facial angiofibroma, shagreen patch, ungual fibroma), brain (cortical tubers, CT; subependymal nodules, SEN; subependymal giant astrocytoma), heart (cardiac rhabdomyoma, CR), retina (hamartomas), lung (lymphangioleiomyomatosis) and kidney (angiomyolipoma) lesions. Identifying a pathogenic variant in *TSC1* or *TSC2* is sufficient for assessing a definite diagnosis, essential for the surveillance and care of individuals and families where clinical criteria are not met[6]. Although they do not constitute diagnostic criteria, seizures, and TSC-associated neuropsychiatric disorders are significant

therapeutic and prognostic stakes in TSC[7]. Epilepsy affects 90% of the patients, with an onset mainly before 24 months of age. It often appears drug-resistant and may affect the neurodevelopmental outcome[8]. In the TOSCA registry, autism spectrum disorder and attention deficit hyperactivity disorder are observed in 21% and 19% of affected individuals, respectively. In comparison, intellectual disability is observed in more than 50%, ranging from mild to profound[9].

In the last decades, a paradigm shift occurred in modalities of diagnosis in TSC. The most common presenting sign was seizures before 2010[10], but now is CRs, which are detected prenatally in 80%[11]. CR is the most common fetal cardiac tumor. It is a benign tumor, generally asymptomatic, which tends to regress spontaneously during pregnancy or the first months of life. Still, it may cause life-threatening issues like arrhythmias and congestive heart failure[12]. In the prenatal period, several other signs of TSC may be detected, such as CTs, SENs, exceptionally subependymal giant astrocytoma, and renal cysts. These signs are of great help in assessing the diagnosis, although none of them are constant[13]. CRs usually appear after 24 weeks of gestation (WG)[14], while neurologic lesions are typically detected later during the late second or third trimester of pregnancy[15]. Without extracardiac signs, it is well known that the prenatal detection of multiple CRs is strongly associated with TSC. However, the diagnostic rate is still interrogated for a unique CR[10,16]. Published data involve a relatively small series of fetuses and indicate diagnosis rates ranging from 10 to 70%[17–19], so estimating the probability of TSC in these cases remains difficult. The significant variability of the disease and the absence of prenatal predictors for the cognitive prognostic, including the detection of CT or SEN[20,21], adds to the difficulty of genetic counseling. An accurate early diagnosis of TSC by clinical or genetic means is crucial for the proper care and support of affected individuals and their families.

Here, we present a retrospective study of the largest cohort of genetic prenatal diagnoses in TSC and a systematic review of the literature to specify the diagnosis rate of TSC in the case of prenatal signs detection.

MATERIAL AND METHODS

1. TSC-suspect fetuses

We retrospectively analyzed the clinical, familial, and molecular data of 561 fetuses who underwent genetic testing for TSC from 2002-2021 in the University Hospital of Angers, France. All fetuses were considered at risk for TSC due to suggestive prenatal signs or familial history of TSC in a first-degree relative. The fetuses were referred by clinicians from 92 centers located in France, Portugal, Switzerland, Romania, and Australia. After receiving professional genetic counseling, all pregnant women gave informed consent for genetic diagnosis of TSC. Fetal samples were obtained through choriocentesis, amniocentesis, and cordocentesis or from various tissues (muscle, lung, CR) collected during fetal autopsy. Whenever possible, blood samples were obtained from both parents, who gave informed consent as per the French legislation. Clinical and familial data were gathered from information forms, medical reports, and letters provided by the prescribing clinician or by consulting the hospital medical file for the fetuses referred locally. This study has been performed in compliance with the Declaration of Helsinki.

2. Genetic analyses

DNA was extracted from fetal and parental samples using appropriate methods and, when necessary, following cell culture of amniocentesis/choriocentesis samples. Maternal cell contamination was excluded for amniocentesis and choriocentesis fetal samples by a PCR-based assay with a set of microsatellites repeat markers (AmpFlSTR Identifiler (Plus) PCR Amplification Kit, 3130xl Genetic Analyzer, GeneMapper software, Thermo Fisher Scientific, Waltham, MA, USA).

From 2002 to 2006, screening for single nucleotide variants (SNV) and small indels was carried out by Denaturing High-Performance Liquid Chromatography (DHPLC). Standard PCR (AmpliTaq Gold, Thermo Fisher Scientific) was used to amplify all the exons and intron-exon junctions in genes *TSC1* and *TSC2*; Amplicons were analyzed by DHPLC (WAVEMaker 3500 HT, ADS Biotec, Omaha, NE, US). When DHPLC detected heteroduplexes, the nucleotidic variation was determined by Sanger sequencing (BigDye terminator v3.1 Cycle Sequencing Kit, 3130xl Genetic Analyzer, SeqScape software, Thermo Fisher Scientific). Next-generation sequencing (NGS) for TSC was introduced in the laboratory in 2016, using the custom IAD49417-133 and IAD62018-185 panels covering all exons and intron-exon junctions of *TSC1* and *TSC2* (Ampliseq Library kit, Ion PI HI-Q Chef kit, Ion PI CHIP kit, Ion Chef, Ion Proton Semiconductor Sequencing System, Thermo Fisher Scientific). Bioinformatic analysis used Torrent Suite (Thermo Fisher Scientific) and NiourK (an analysis pipeline developed in-house). Sanger was then used as a confirmation technique for variants identified by NGS.

From 2007, a negative result for DHPLC/Sanger/NGS analysis was followed up with screening for large deletions or duplications in *TSC1* and *TSC2* by Multiplex Ligation-dependent Probe Amplification (MLPA). SALSA MLPA kits P124 TSC1, P337 TSC2, and (from 2010) P046 TSC2 (MRC Holland, Amsterdam, NL), and fragment analysis by capillary electrophoresis (3130xl Genetic Analyzer, GeneMapper software, Thermo Fisher Scientific, Coffalyser software, MRC Holland) were used.

Investigation of a known familial variant in *TSC1* or *TSC2* was performed by targeted DHPLC/Sanger sequencing for SNV/small indels and by a specific MLPA kit for structural variations (SV).

Variants in *TSC1* and *TSC2* are described using the reference transcripts NM_000368.5 and NM_000548.5, respectively. All the variants were transmitted to the LOVD database curators[22].

3. Systematic review of the literature

A systematic search of the English literature was performed in August 2023 using MEDLINE (Pubmed), using the keywords "(prenatal OR antenatal OR gestational) AND (tuberous sclerosis OR rhabdomyoma)." We included all publications presenting prenatally diagnosed CR. The exclusion criteria were (i) case reports of < 2 individuals, (ii) cohort including only TSC-confirmed individuals, and (iii) lack of information to assess the clinical or genetic diagnosis of TSC in the unique CR versus multiple CR groups.

4. Diagnosis assessment

The clinical diagnosis was assessed for individuals in this study and from the literature based on the available data, using the latest diagnostic criteria edited by H Northrup *et al*. in 2021[6]. Genetic variants were classified using ACMG-AMP criteria[23] and ClinGen SVI group refinements[24–27]. Details about how these criteria were applied are provided (Supplementary Table SI). When available in the literature, genetic data was kept as per the authors' interpretation.

RESULTS

1. Clinical data

Excluding 321 fetuses that had a targeted test for a familial history with an identified genetic variant, this series includes 240 fetuses tested for signs suggestive of TSC (Table I, Supplementary Table SII). All the fetuses had CR detected at a mean term of 25.2 WG, ranging from 14.0 to 36.9 WG. One hundred fifty-eight fetuses harbored multiple CRs, and 82 presented with a unique CR at first detection. Among these, 40.2% (33/82) were found to have at least one more CR later in pregnancy. Seven were ultimately suspected of having a differential diagnosis: cardiac fibroma (fetuses 11, 34, 71, 96, 185) or malformation (fetuses 58, 188). Cardiac complications were associated at first detection in two (fetuses 76, 236), and fetal hydrops was reported later in pregnancy in four (fetuses 79, 152, 196, 227). In four fetuses, CR was not detected prenatally or was not the first sign. Two fetuses (fetuses 10, 91) presented with fetal death *in utero* (FDIU), while no ultrasound sign was detected prenatally. TSC-suggestive lesions were detected by autopsy. Two had prenatal atypical neurologic lesions (fetus 49: unilateral cerebellar dysplasia, multiple supratentorial heterotopias; fetus 189: isolated ventriculomegaly, then hemimegaloencephaly). The test was conducted after *post-mortem* detection of cardiac and neurologic lesions.

CT or SEN were found in 25.6% (21/82) and 49.4% (78/158) of the fetuses with an initial diagnosis of unique CR and multiple CRs, respectively. They were first detected mainly by fetal cerebral MRI and ultrasound, four weeks after the CRs on average (29.3 ± 3.2, range 20.1-35.0 WG), including CTs and SENs identified by autopsy. In five, they were seen since the initial detection of CR (fetuses 43, 111, 119, 206, 223) and after a livebirth in two (fetuses 69, 141). Renal cysts were reported in nine.

At least 161 couples opted for termination of pregnancy (TOP), and five fetuses died *in utero*. During autopsy or after a live birth, cutaneous signs were noticed, with 13 individuals presenting with achromic macules and five with cutaneous hamartomas. Among the 27 liveborn fetuses, postnatal clinical information was available in 16 infants with a maximal follow-up of 30 months, and eight of them developed other signs of TSC. Epilepsy or abnormal EEG was detected in five (fetuses 68, 69, 138, 141, 204), developmental delay or intellectual disability in two (fetuses 68, 204), and retinal hamartomas in one (fetus 204).

Ultimately, a definite clinical diagnosis could be assessed prenatally in 18.3% (15/82) and 36.7% (58/158) in the unique CR and multiple CR groups, respectively. The clinical diagnosis rates rose to 23.1% (19/82) and 48.1% (76/158) in the same groups when counting postnatal diagnoses (after live birth or autopsy).

2. Genetic results

We identified 142 unique variants in *TSC1* (n=17) and *TSC2* (n=125), distributed in 172 fetuses from 171 unrelated families. Fetuses 194 and 195 were affected siblings; the former was not tested initially, but a DNA sample was extracted during the autopsy, and both were tested simultaneously after signs appeared in the latter. No variant was identified in 68 fetuses.

In 156 fetuses with pathogenic variant (ACMG class 4-5), the most frequent findings were *TSC2* missense (n=38) or frameshift (n=34) variants, typically occurring *de novo* (Figure 1). Twenty-two pathogenic variants were inherited. A familial investigation indicated a parental possible or definite clinical diagnosis in 10, and this information was decisive in the confirmation of the diagnosis in three (fetuses 67, 102, 165). Searching through the local and public databases, finding the variant in at least four unrelated index cases without

healthy carriers led to the assessment of the variant as pathogenic in three (fetuses 102, 157, 165). Two were diagnosed with two pathogenic variants (fetuses 173, 215).

Eighteen variants were classified as variants of unknown significance (VUS). In fetus 143, a *de novo* missense (*TSC2*:c.5111C>T, p.(Ser1704Phe)) was found. In fetus 124, a large duplication, including exons 5 to 28 of *TSC2,* was identified. It could be reclassified as likely pathogenic if proven *de novo,* but unfortunately, no paternal sample was available. All other VUS were inherited from a parent, three of them having a possible or definite TSC diagnosis after checkup (fetuses 142, 153, 198). Demonstrating a *de novo* character in these affected parents would shift their respective variants assessment to pathogenic or likely pathogenic. Lack of clinical data or a negative familial investigation prevented us from concluding the remainder.

In total, a definite genetic diagnosis was made in 46.3% (38/82) of fetuses in the unique CR group and 74.7% (118/158) in the multiple CR group (Figure 2).

Including all the fetuses tested prenatally in our center, a prenatal diagnosis was conducted for fetuses of 114 unrelated families where an offspring carried a *de novo* variant in *TSC1* or *TSC2*. We report 3 cases of germinal mosaicism, 2 of which were demonstrated with both maternity and paternity confirmed (fetuses 141, 218). In the last family, a man had an affected fetus with TOP from a first union. No genetic test was conducted at that time. A pathogenic variant (*TSC2*:c.1257+1G>, p.?) was found in the second affected fetus (fetus 42) conceived from a new union but not in his parents, indicating probable germinal mosaicism of paternal origin. Thus, this series' risk of germinal mosaicism is 2.6% (3/115).

3. Systematic Review of the literature and final overall diagnoses

A total of 36 publications were retained (Figure 3), and the clinical and molecular data of 387 individuals with prenatally diagnosed CR were analyzed (Supplementary Table SIII). The

selected studies provided data about 118 fetuses with a unique CR and 269 fetuses with multiple CRs. The overall diagnosis rate, including clinical and genetic confirmation, was 32.2% (38/118) with unique CR and 74.7% (201/269) with multiple CR. In the 72 fetuses where a definite clinical diagnosis was assessed and a genetic test was performed, 10 (13.9%) had no variant identified (NVI) or carried a VUS, according to the authors (Table II).

DISCUSSION

We describe the most extensive series of prenatal diagnoses in TSC, regarding both clinical and molecular data, adding arguments to specify the risk of TSC in case of prenatal detection of unique or multiple CRs.

Detecting a prenatal CR is a challenging situation for the estimation of fetal prognosis, especially when the CR is unique and isolated. Although the tumor itself is generally not life-threatening, it raises suspicion about its potential association with TSC, characterized by great variability and a significant risk of neurocognitive disorders[9]. Given the possibility of developing severe disease, according to French legislation, a TOP upon parents' request is usually considered acceptable. Recently, treatments based on mTOR inhibitors and antiepileptic drugs have been experimented on postnatally for preventive purposes[28–30] or *in utero* for symptomatic use[31,32] and may reduce the severity of the disease.

In this series, the final diagnosis rate was 80.3% in the multiple CR group, including clinical or molecular diagnoses, consistent with a rate of 75-80% in the literature[7]. In the unique CR group, we observed a higher diagnosis rate of 50.0% compared to 32.2% in published cases. This is likely since we defined our groups based on the first CR detection, whereas this information is rarely reported in the literature. When excluding the 33 fetuses in whom at least one other CR was secondarily detected, our final diagnosis rate is 30.6% (15/49), which is very comparable to that of the literature. This is interesting novel data because the CR is initially unique and isolated in roughly one-third of the cases, and the fetal prognosis is already questioned at this step.

Since the first reported association of prenatal CR with TSC[33], the shift towards early and prenatal diagnoses[11] reflects advances in prenatal imaging and the molecular field. Although they have different imaging characteristics, other fetal cardiac tumors may be mistaken for

CR, the most frequent case being the confusion between a unique CR and a fibroma. Fibromas are solitary, homogeneous masses less echogenic than CR that may be calcified[12,34]. In this series, cardiac fibromas were presumed in five (fetuses 11, 34, 71, 96, 185). In all cases, there was no sign of TSC. The genetic test was negative, and the babies were liveborn. A postnatal ultrasound follow-up was available in three, and two regressed completely. Teratomas are usually easier to distinguish from CR because of their heterogeneous aspect with cystic components. Still, a multilobulated teratoma might look like multiple CR, as reported by Zhou *et al.*[35]. Cardiac malformations may also mislead the sonographer in the early stages of the pregnancy, as in fetus 58, where a doubt between CR and septal aneurism at 14 WG led to a prenatal genetic test for TSC, which proved negative. In fetus 188, an isolated CR was seen prenatally; the genetic test was negative, but a TOP was performed due to cardiac complications. Autopsy demonstrated a complex cardiac malformation without CR; this might be a differential diagnosis but does not exclude an accurate initial diagnosis followed by CR regression. In the case of authentic CR, even though multiple lesions are strong predictors of TSC, multiple CRs have been reported in a case of Pompe disease with negative *TSC1*/*TSC2* investigations[36], as well as in one case of Beckwith-Wiedemann[37], and Prader-Willi syndromes[38]. However, a coincidence or a double diagnosis cannot be excluded from these unique reports[39].

Given the natural history of the disease and the diagnostic criteria requirements, the detection of CNS lesions associated with CR is the only way to prenatally assess a definite clinical diagnosis of TSC using the postnatal criteria. Polycystic kidneys might help guide the diagnosis, but this sign is rarely observed and constitutes only a minor criterion. CTs and SENs are inconstant and tend to be detected late in pregnancy; their absence cannot exclude the diagnosis. However, finding these characteristic lesions in a fetus with CR is not always sufficient: diagnostic criteria require at least two SENs or multiple CT / radial migration lines

to constitute a major criterion[6]. According to the criteria, TSC was not clinically confirmed in six fetuses of this series, presenting only a unique CT (fetuses 80, 193), a unique SEN (fetuses 82, 155), or even one of each (fetuses 73, 236). A pathogenic variant was found in *TSC1* or *TSC2* in all. This underlies the difficulty of confirming TSC in the prenatal period based on the absence of prenatal diagnostic criteria. Current diagnostic criteria were tailored to have excellent specificity in an adult population but lack sensibility in young children and even more in fetuses where very few signs of the disease are penetrant. This study might provide informative data to help build clinical criteria adapted for prenatal diagnosis in TSC.

On the genetic level, the evolution of techniques with the successive introduction of DHPLC[40], MLPA[41], and NGS[42] in the postnatal and prenatal tests offered more comprehensive analyses for *TSC1* and *TSC2* genes. Furthermore, the 2015 ACMG-AMP criteria[23] provided a framework that helped harmonize the variants' interpretation. Pathogenic variants in this series were located in *TSC2* in 91% (143/158), more than the 74% reported in the general TSC population[7]. Given that *TSC1* variants are more frequently found in familial cases[7], this skewed *TSC2*/*TSC1* ratio was expected in our series, which excludes known familial variants. Together, nonsense and frameshift are the most frequent types of pathogenic variants but should still be assessed cautiously, for it is now well-demonstrated that they are not always deleterious. They may occur in healthy individuals in the alternatively spliced exons 26 and 32 of *TSC2*[43]. No pathogenic variant has been described in these exons, which are absent from most transcripts in adult tissues. Another recent example is the non-penetrant frameshift variant *TSC2*:c.4255_4256del, which creates a cryptic splice site, resulting in an in-frame deletion that retains the TSC2 function[44].

However, the most challenging task remains the interpretation of missense variants and inframe indels, especially when they are inherited. A well-conducted checkup, including dermatologic, ophthalmologic examination, and brain and kidney imaging, is essential to

determine the status of the relatives carrying the variant and can be decisive for the variant assessment. Finding known pathogenic variants in completely asymptomatic relatives raised important questions about penetrance, with the tremendous intra-familial variability in age of onset and severity known in TSC and genetic counseling. For example, the *TSC2*:c.1229T>G, p.(Leu410Arg) pathogenic missense variant was detected in fetuses 57 and 149. It is absent from control population databases, and bioinformatic tools strongly predict a functional impact. *In vitro* tests demonstrated an impaired ability to inhibit mTOR activity compared to wild-type *TSC2*[45]. In both cases, it was inherited from a parent who showed no sign of TSC after a complete checkup (Figure 4A, 4B). Another example is the *TSC2*:c.1283_1285del, p.(Ser428del) pathogenic variant, which presents a significant but low impact in functional tests[46]. This variant was observed in five fetuses of this series: *de novo* (fetus 27, Figure 4C) or inherited from a parent with a history of epilepsy or isolated renal cysts (fetuses 46, 173, 194-195, Figure 4D-4F). Only the fetuses fulfilled the criteria for a possible or definite clinical diagnosis of TSC, while this was not the case in four adult relatives, including one with a complete checkup. The familial data in these cases challenges variants interpretation and could radically change genetic counseling. While functional tests are of great help for interpretation, they might not always accurately capture the biological phenomena occurring *in vivo*, and it seems essential to refer to clinical data first whenever available.

Two fetuses were found with two pathogenic variants. Fetus 173 carried the maternally inherited *TSC2*:c.1283_1285del, p.(Ser428del) discussed above, and the pathogenic *de novo* *TSC2*:c.3418_3419=/insACTG, p.(Gly1140=/Aspfs*29) in the mosaic state. Since biallelic pathogenic variants in TSC2 are nonviable[48], either both variants occurred in *cis* on the maternal allele, or the mosaic variant occurred in *trans* but with an allele frequency very low and null in critical tissues. Fetus 215 carried the pathogenic nonsense *TSC1*:c.891T>G, p.(Tyr297*) and the recurrent missense *TSC2*:c.1831C>T, p.(Arg611Trp), both of which

appeared *de novo*. The latter has a well-described functional impact[47] and is reported as pathogenic many times in our series and databases (LOVD: TSC2_000053; ClinVar ID: 49643). To our knowledge, this is the first report of germline pathogenic variants in both *TSC1* and *TSC2*. Germline *de novo* hits in two loci responsible for the same disease is a highly improbable event but theoretically compatible with life.

Finally, as shown by the nearly 15% of fetuses with definite clinical diagnoses having a negative genetic test in our series and the literature, there is room for improvement in molecular diagnosis of TSC. Negative tests in such cases may be explained by the inability of current routine techniques to identify specific variants, the fact that not all regions are sequenced or interpreted, and technical or bioinformatic flaws. Few studies have explored this issue in the context of TSC[49,50]. It follows that intronic, low-mosaic, and somatic variants in *TSC1* and *TSC2* seem to explain most cases of TSC, with no variant identified by conventional techniques.

To conclude, we present the most significant series and a systematic review of the literature on fetuses with prenatally detected CR. The initial detection of a unique CR led to a definite TSC diagnosis in half of the cases, and multiple CRs were associated with TSC in 80%. We emphasize the need for complete fetal and familial phenotyping and the association of clinical and molecular data to assess accurate variant interpretation and genetic counseling. Early diagnosis helps guide clinicians and families to make better-informed decisions in case of prenatal CR detection. In the future, it might also change the natural history of the disease through therapeutic advances.

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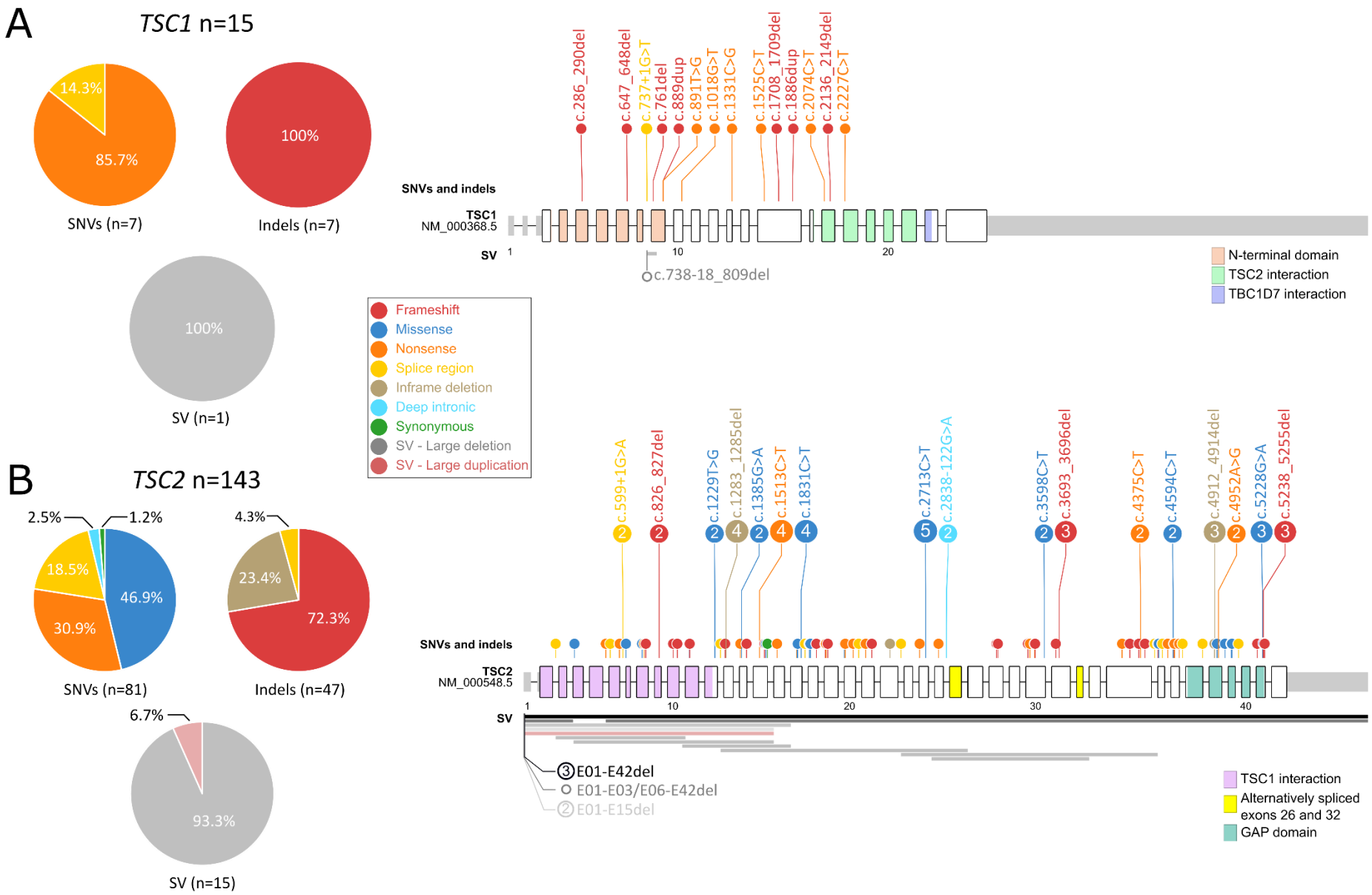

Figure 1: Characteristics of the pathogenic variants identified in fetuses referred to prenatal signs suggestive of TSC

Left panel: diagrams presenting the predicted effect of single nucleotide variants (SNV, top left), indels (top right), and the type of structural variants (bottom) identified in *TSC1* (A) and *TSC2* (B). Right panel: representation of *TSC1* and *TSC2* variants on their respective mRNA. Exons are represented as white boxes, and introns are represented as lines. All variants are depicted in *TSC1,* whereas only recurrent variants are detailed in *TSC2*. Deletions and insertions are categorized as "indels" if < 50b and structural variants (SV) if ≥ 50b. Variants affecting an intron in position ±5 of an exon are annotated "Splice region." Beyond the ±5 nucleotides, a variant is considered "Deep intronic." The numbers inside the circles indicate the number of unrelated families a variant was identified in. The protein functional domains and the predicted consequence of each variant are depicted by a color code, as shown in the legend.

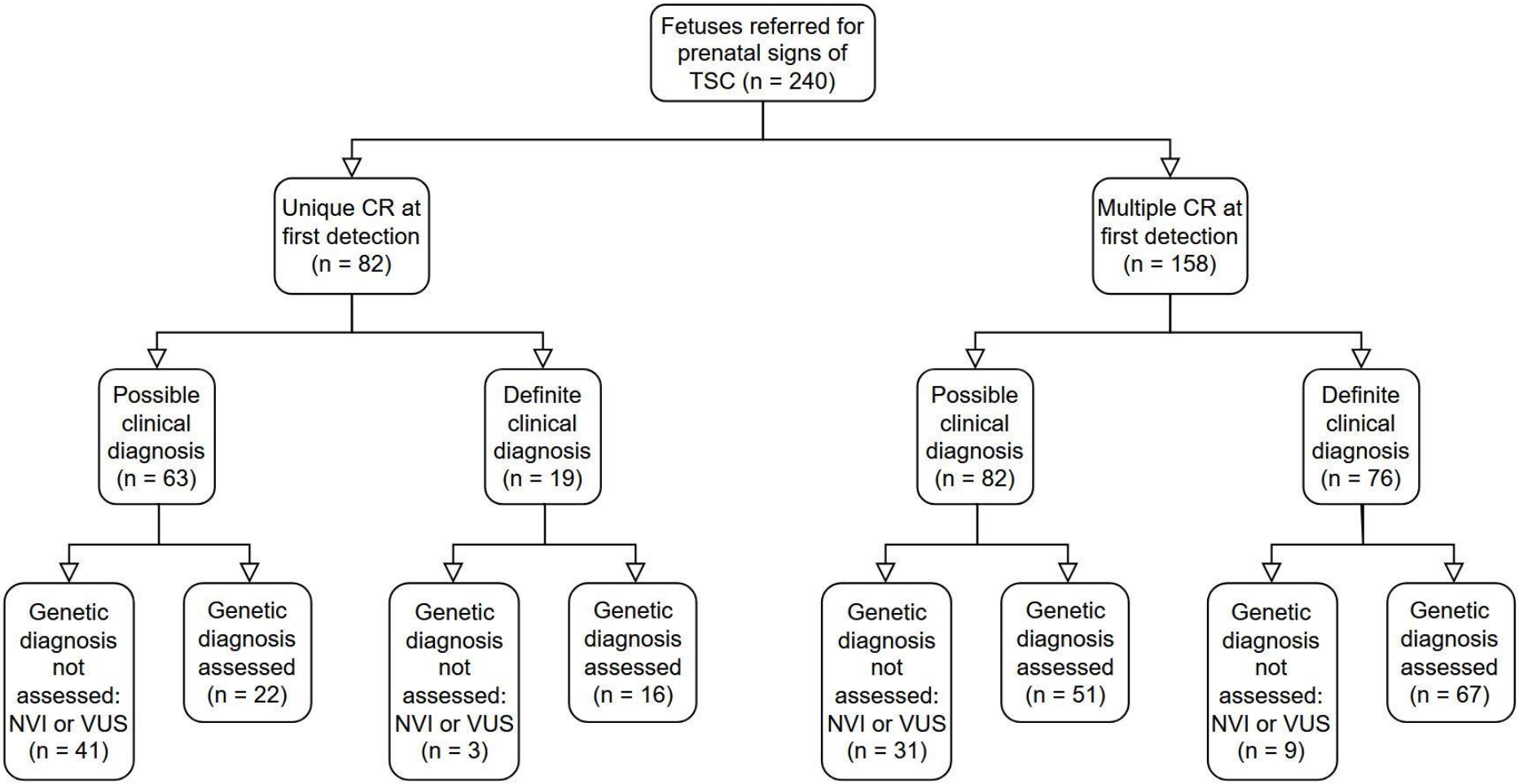

Figure 2: Chart of the main clinical and molecular outcomes of fetuses referred to prenatal signs suggestive of TSC

CR, cardiac rhabdomyoma; NVI, no variant identified; VUS, variant of unknown significance.

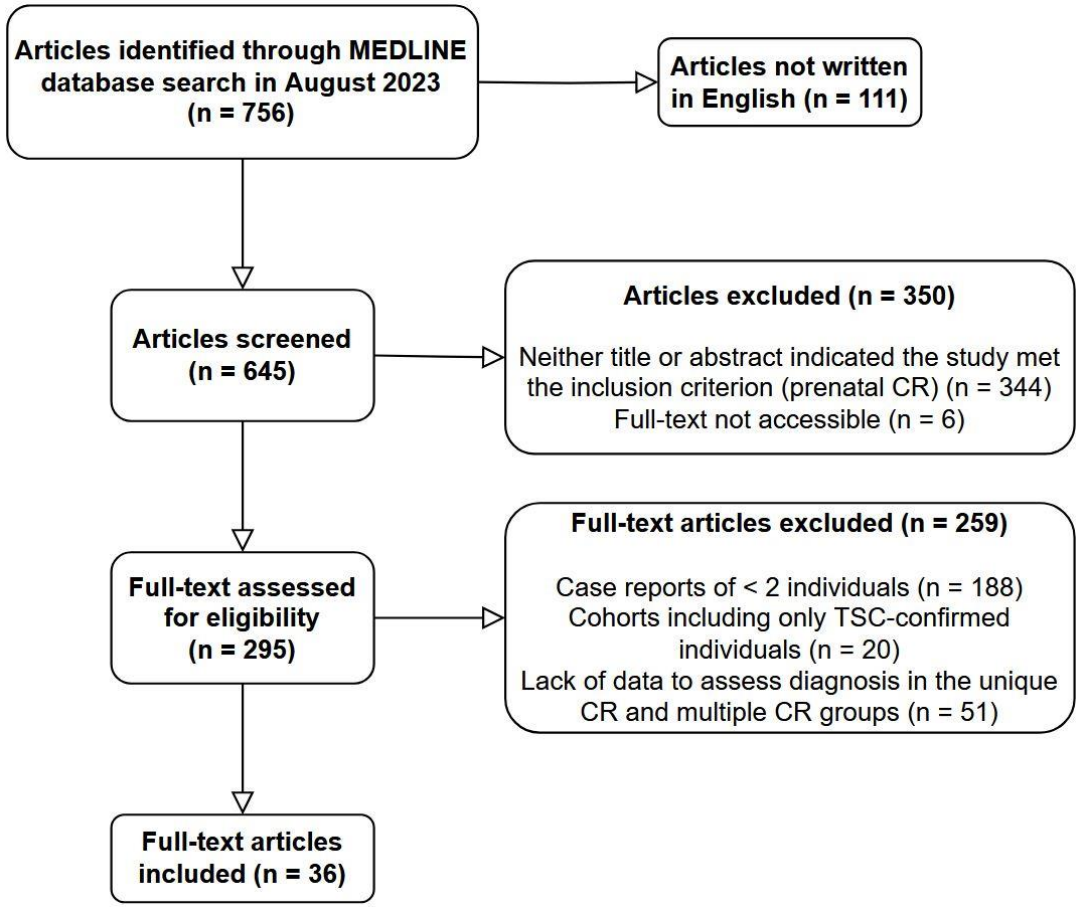

Figure 3: Flowchart of the systematic review of the literature

The MEDLINE search was performed with the keywords "(prenatal OR antenatal OR gestational) AND (tuberous sclerosis OR rhabdomyoma)." CR, cardiac rhabdomyoma.

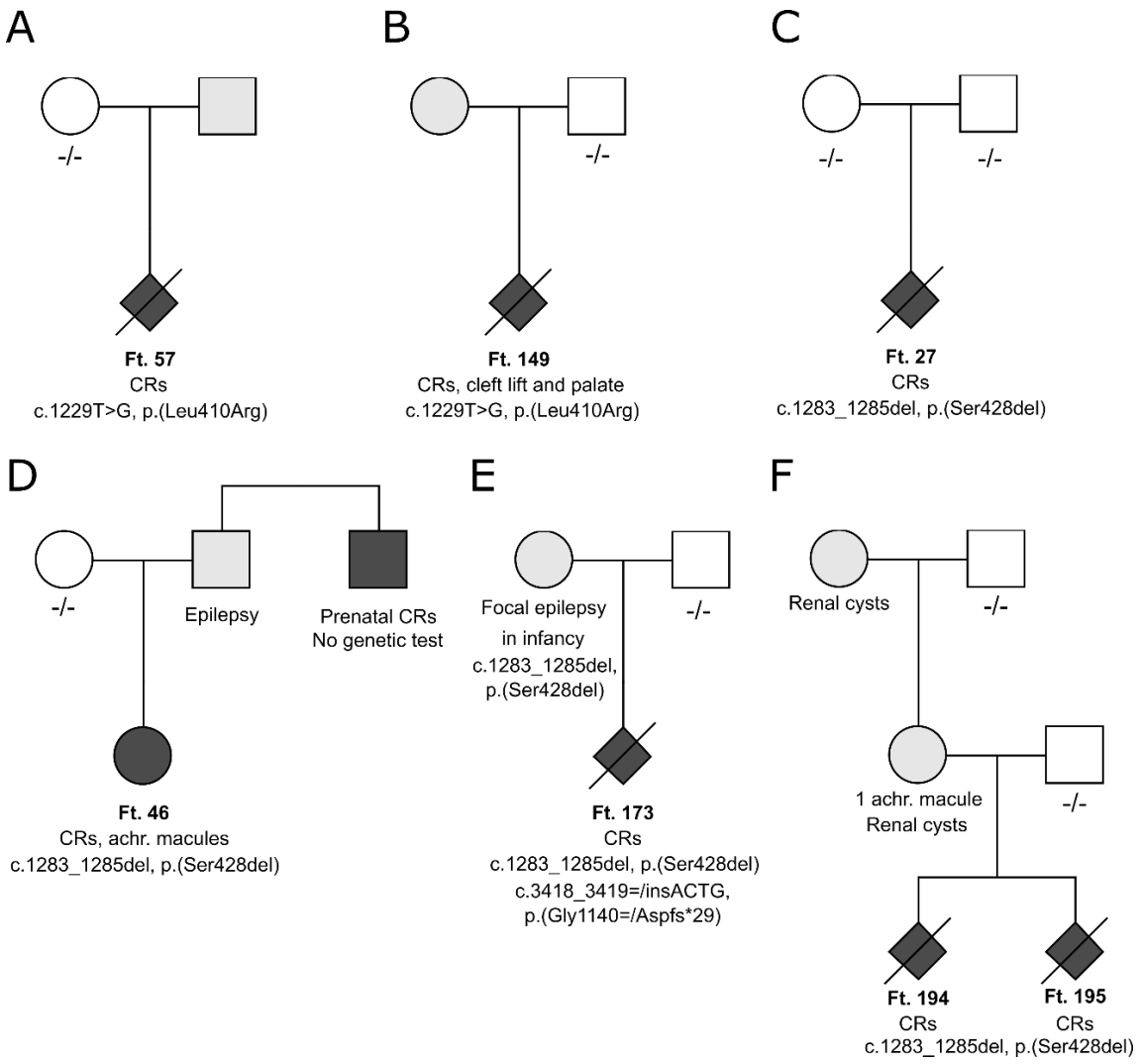

Figure 4: Pedigrees of some families comprising healthy carriers of pathogenic variants in *TSC2*

Males are represented as squares, females as circles, and fetuses of unknown sex as rhombs. Solid black figures represent individuals with a clinical "Possible" or "Definite" diagnosis; solid light grey indicates an unaffected carrier. Tested individuals not carrying the variant are shown by "-/-."

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Table I: Clinical characteristics of the fetuses referred for TSC-suggestive prenatal signs (n=240)

Characteristic	Value
Fetal sex	Male (n=84) Female (n=67) Unknown (n=89)
Sample type	Amniotic fluid (n=147) Fetal blood (n=52) Lung (n=12) Muscle (n=6) Chorionic villus (n=5) Postnatal blood (n=3) CR (n=2) Tissue not specified (n=29) Tested in a second tissue (n=16)
Known familial history of TSC	Affected relative, no genetic test (n=3) Affected relative, no variant identified (n=1)
Gestational age	First sign: 25.2 ± 4.9 (14.0-36.9) WG First CNS sign: 29.3 ± 3.2 (20.1-35.0) WG
Outcome of pregnancy	TOP (n=161): 30.4 ± 3.9 (21.7-38.0) WG Birth (n=27): 36.9 ± 2.1 (32.2-39) WG FDIU (n=5): 28.0 ± 6.1 (20.1-36.9) WG
Fetopathology	n = 90
First sign	Isolated CR (n=228) CR and other signs (n=8): CT (n=4), SEN (n=1), cardiac decompensation (n=2), subependymal cysts (n=1) FDIU (n=2) Atypical CNS signs (n= 2)
CR number (first detection)	Multiple CRs (n=158) Unique CR (n=82), including secondary multiple CRs (n=33)
CR main localization (first detection)	LV (n=94) RV (n=37) Septum (n=29) RA (n=6) Apex (n=6)
CR maximal size (first detection)	13.3 ± 9.0 (4-60) mm
Fetal MRI	n=104 30.0 ± 2.8 (22-35.6) WG
CT	Multiple (n=75) Unique (n=8)
SEN	Multiple (n=65) Unique (n=8)
CNS lesion's first detection methods	Fetal MRI (n=58) US (n=23) Autopsy (n=14) Postnatal imaging (n=3)
Other findings	CNS: heterotopia and neuronal migration disorders (n=4), cortical dysplasia (n=3), isolated ventricle dilatation (n=3), astrocytoma (n=1), hemimegaloencephaly (n=1), subependymal cyst (n=1), choroid plexus cyst (n=1), septum pellucidum cyst (n=1), abnormal frontal gyration (n=1), colpocephaly (n=1) Skin: achromic macule(s) (n=13), hamartoma (n=4), verrucous nevus (n=1) Kidney: renal cysts (n=9), hydronephrosis (n=2), renal agenesis (n=1), pyelic and ureteral duplication (n=1), undefined nodule (n=1) Lung: abnormal lobulation (n=2), undefined tumours (n=1) Retinal hamartomas (n=1) Growth: IUGR (n=3), overgrowth (n=2) Various malformations and malformative associations (n=4)

Table II: Clinical, genetic, and overall diagnoses in this series and in the literature review

Parameter	This series	Review
Definite clinical diagnoses in the unique CR group	23.1% (19/82, including 15 prenatal)	22,3% (23/103)
Definite genetic diagnoses in the unique CR group	46.3% (38/82), plus 9 VUS	35,5% (27/76), plus 3 VUS
Overall diagnoses in the unique CR group	50.0% (41/82) Subtracting secondary CRs: 30.6% (15/49)	32,2% (38/118)
Definite clinical diagnoses in the multiple CR group	48.1% (76/158, including 58 prenatal)	49,6% (116/234)
Definite genetic diagnoses in the multiple CR group	74.7% (118/158) plus 7 fetuses with one or more VUS	75,1% (136/181) plus 15 VUS
Overall diagnoses in the multiple CR group	80.3% (127/158) Adding secondary multiple CRs: 80.1% (153/191)	74,7% (201/269)
Negative genetic test (NVI or VUS) among definite clinical diagnoses	12.6% (12/95)	13.0% (9/69)

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SUPPLEMENTARY MATERIALS

1. Supplementary Table SI: ACMG-AMP Criteria Application

Crite-rion	Description (ACMG/AMP 2015)	ClinGen SVI updated reco.	Application in this study
BA1	Allele frequency is above 5% in Exome Sequencing Project, 1000 Genomes, or ExAC.	Ghosh 2018[1]	
BS1	Allele frequency is greater than expected for the disorder.		Threshold for BS1 has been set at 0.1% (maximum allele frequency in a substantial control population, i.e., >1.000 individuals)
BS2	Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance expected at an early age		NA: not fully penetrant at an early age
PM2	Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes or ExAC	Unpublished[2]	As per the updated recommendation, PM2 is applied with the strength "supporting." The combination PVS1+[pathogenic supporting] classifies a variant as "Likely pathogenic."
PS4	The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls.		This rule was applied only for VUS variants, which might be reclassified as likely pathogenic or pathogenic based on this argument. Probands were searched in the LOVD and Clinvar databases, in the literature, and in our local database. Individuals present in several of these sources were systematically searched to avoid double counting. *In the LOVD database, summary records and classification records were not counted as probands. Clinvar entries were counted only if the condition was specified to be TSC or the phenotype could be gathered from the submitter. The search for the variants in the literature was performed using the Mastermind Genomic Search Engine (https://www.genomenon.com/mastermind).* Since no official recommendation has been emitted by TSC experts for this criterion, PS4 was applied as follows: - PS4_Strong: variant observed in ≥ 8 probands from different families and absent in unaffected individuals (from control databases or relatives of the tested fetus) - PS4_Moderate: variant observed in ≥ 4 probands from different families and absent from control databases (from control databases or relatives of the tested

			fetus)
BP1	Missense variant in a gene for which primarily truncating variants are known to cause disease		NA
BP3	In-frame deletions/insertions in a repetitive region without a known function		
BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site, AND the nucleotide is not highly conserved.		**Missense variants**: 2 predictors (CADD and REVEL) were used, and PP3/BP4 rules were applied with different strengths in accordance with thresholds set by the ClinGen 2022 recommendations. *When both predictors suggested pathogenicity or no impact with discordant strength, the result with the weakest strength was applied.*
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.)	Pejaver 2022[3]	*When one predictor suggested pathogenicity, and the other no impact, neither BP4 nor PP3 were applied.* *When the CADD score fell in the pathogenic "moderate" category (maximum for this predictor) and the REVEL score in the pathogenic "strong" category, PP3 was applied with strength "strong."*
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc...)	Pejaver 2022[3]	**Splice-region, synonymous, and intronic variants** not fulfilling PVS1 criterion: 2 predictors (SpliceAI and dbscSNV ADA or SpliceAI and SPiP) were used. *When one predictor suggested pathogenicity (spliceAI >0.5, dbscSNV >0.7, or SPiP > 0.9), PP3 was applied.* *When both predictors suggested no splicing impact (spliceAI <0.1 and dbscSNV <0.15 or spliceAI <0.1 and SPiP <0.1), BP7 was applied.* *In any other case, neither BP7 nor PP3 was applied.*
PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants.		
PM5	Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before		
PS1	Identical amino acid change as a previously established pathogenic variant regardless of nucleotide change		
PVS1	Null variant (nonsense, frameshift, canonical +/−1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function (LOF) is a known mechanism of disease	Abou Tayoun 2018[4]	
BS3	Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing.	Brnich 2020[5]	
PP2	Missense variant in a gene that has a low rate of		NA

	benign missense variation and where missense variants are a common mechanism of disease		
PM1	Located in a mutational hot spot and/or critical and well-established		NA
PS3	Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product	Brnich 2020[5]	
BS4	Lack of segregation in affected members of a family		
PP1	Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease		Applied if the relatives carrying the variant fulfill the criteria for a possible or definite TSC diagnosis
PM6	Assumed de novo, but without confirmation of paternity and maternity	Unpublished[6]	
PS2	De novo (both maternity and paternity confirmed) in a patient with the disease and no family history	Unpublished[6]	Applied for variants found in mosaic state in fetuses since the variant is necessarily post-zygotic. Also applied to fetal heterozygous variants inherited from a parent carrying the variant in a mosaic state.
BP2	Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern		
PM3	For recessive disorders detected in trans with a pathogenic variant	Unpublished[7]	NA
BP6	A reputable source recently reported variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation.	Biesecker 2018[8]	Applied if a majority of Clinvar interpretations are Benign / Likely Benign
PP5	A reputable source recently reported variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation.	Biesecker 2018[8]	Applied if Clinvar interpretation is Pathogenic / Likely pathogenic and review status is at least 2 stars (criteria provided, multiple submitters, no conflicts)
BP5			
PP4			Applied if the fetus or a first-degree relative fulfills the criteria for a definite TSC diagnosis

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2. Supplementary Table SII: Clinical and molecular data

Attachment: **Supplementary Table SII: Clinical and molecular data.xlsx**

3. Supplementary Table SIII: Literature review

Study	CR	Clinical DD	TSC1/2 variant (P/LP)	TSC1/2 variant (P/LP + VUS)	Clinical or genetic DD	Clinical DD, NVI ou VUS*	Comments
Zhai 2023[1]	11 S 24 M	1/11 8/24	1/6 7/12	1/6 10/12	1/11 10/24	0/1 1/6	
Santana 2023[2]	2 S	1/2	NA		1/2	-	Case 1: unique SEN Case 2: multiple SEN and probable unique CT
Malinger 2023[3]	4 S 15 M	0/4 11/15	2/3 7/9		2/4 12/15	- 1/7	Case 8: number of SEN not specified, one achromic macule at 7 years of age, no genetic test Case 11: "white matter lesions" Case 16: single CT, positive genetic test Cases 1, 13, 18, and 19: multiple CT, number of SEN not specified
Jin 2023[4]	19 S 20 M	2/19 8/20	2/13 9/18		2/19 9/20	0/2 0/8	One proven fibroma excluded, assumed negative for any TSC sign Case 36: unique SEN Paraventricular lesions are assumed to be SEN, white matter, and parenchymal lesions as CT.
Gamboa 2023[5]	1 S 2 M	1/1 2/2	NA NA		1/1 2/2	- -	
Okmen 2022[6]	3 S 11 M	1/3 8/11	NA 1/1		1/3 9/11	- -	
Atiq 2022[7]	1 S 1 M	1/1 1/1	NA NA		1/1 1/1	- -	
Wang 2021[8]	5 S 3 M	0/5 0/3	4/5 2/3		4/5 2/3	- -	6 fetuses with no variant were identified initially, and 2 suspected of parental gonadal mosaicism Tests were performed on umbilical cord blood and CR tissue by hybrid-capture-based NGS and digital droplet PCR Low-level mosaic variants in umbilical chord were considered to fulfill TSC genetic diagnosis, but not somatic variants found only in CR.

Qi 2021[9]	18 S 43 M	3/18 25/43	4/18 27/43	7/18 38/43	4/18 33/43	0/3 7/25	Case 23: positive prenatal MRI, lesions not specified Case 33: 2 *de novo* VUS in *TSC2*, suspected parental germinal mosaicism (third pregnancy with a fetus presenting CR) Case 45: 2 variants identified (TSC1 LP / TSC2 VUS)
Pavlicek 2021[10]	1 S 8 M	0/1 5/8	NA 8/8		0/1 8/8	 0/5	
Zhen 2020[11]	1 S 14 M	0/1 0/14	1/1 10/10		1/1 10/14	- -	
Ulm 2020[12]	9 S 4 M	6/9 2/4	6/9 4/4		6/9 4/4	0/6 0/3	Case 14: proven fibroma excluded
Behram 2020[13]	2 S 16 M	0/2 0/16	0/2 10/16		0/2 10/16	- -	
Mariscal-Mendizábal 2019[14]	5 S 5 M	2/5 1/5	2/2 1/2		4/5 2/5	- -	Case 4: "cortical tubes in 3rd ventricle" assumed as multiple SEN
Chen 2019[15]	15 S 35 M	5/50	5/15 32/35		5/15 32/35	- -	CNS information could not be linked to unique or multiple CR cases, and clinical and genetic diagnoses could not be linked. Clinical diagnosis excluded from the final counts in the single and multiple CR groups. Cases 2 and 45: proven fibroma and hemangioma excluded
Gu 2018[16]	2 S 13 M	0/2 0/13	0/2 11/13	0/2 12/13	0/2 11/13	- -	
Sciacca 2014[17]	1 S 9 M	1/1 9/9	NA NA		1/1 9/9	- -	
Lee 2013[18]	4 S 13 M	1/4 8/13	4/NA		1/4 8/13	- -	Unable to specify the number of genetic tests passed, the repartition of positive genetic tests, nor the repartition of clinical signs between unique and multiple CR groups. Genetic diagnoses were excluded from the final counts in single and multiple CR groups. Clinical and genetic diagnoses were fully concordant.
Nemec	4 S	3/4	NA		3/4	-	

2012[19]						
Dereddy 2008[20]	3 M	3/3	NA	3/3	-	
Mühler 2007[21]	1 S	0/1	NA	0/1	-	No post-natal follow-up. Maternal genetic test negative (child not tested)
	5 M	4/5	4/4	5/5	-	
Lacey 2007[22]	1 S	0/1	NA	0/1	-	
	2 M	1/2	NA	1/2	-	Case 3: unique CT
Gazit 2007[23]	3 S	0/3	NA	0/3	-	
Jóźwiak 2006[24]	5 M	5/5	1/1	5/5	0/1	
Chen 2005[25]	2 M	2/2	2/2	2/2	0/2	Case 1: single SEN Case 2: single CT, multiple SEN
Nir 2001[26]	3 M	3/3	NA	3/3	-	Case 3: unique retinal hamartoma
Bonnamy 2001[27]	1 S	0/1	NA	0/1	-	
	2 M	2/2	NA	2/2	-	
Di Liang 2000[28]	2 M	1/2	NA	1/2	-	"The diagnosis of TS was based on the presence of both cutaneous stigmata (hypopigmented maculae, facial angiofibroma, or shagreen patch) and hamartomatous malformations of the central nervous system."
Sonigo 1996[29]	7 M	5/7	NA	5/7	-	Case 5: single SEN Case 8 excluded: "one myoma" in the table, but fetuses were all supposed to have multiple CRs according to the abstract.
Itoh 1996[30]	2 S	0/2	NA	0/2	-	
Komori 1995[31]	2 S	0/2	NA	0/2	-	Case 1: second CR and limb malformations identified by autopsy
Werner 1994[32]	2 M	2/2	NA	2/2	-	
Bordarier 1994[33]	1 S	0/1	NA	0/1	-	
	2 M	2/2	NA	2/2	-	White matter or periventricular nodules made of balloon cells are assumed to be CT or SEN equivalents, respectively.
Wallace 1990[34]	1 S	1/1	NA	1/1	-	
	3 M	2/3	NA	2/3	-	Case 2: "Two areas of decreased attenuation in

							the frontal horns that were similar to the changes of resolving infarction" assumed as multiple SEN; a calcified SEN was later described. Case 5: 2 achromic macules at age 5 months do not fulfill the criteria
Chitayat 1988[35]	2 M	1/2	NA		1/2	-	Case 2: unique SEN
Muller 1986[36]	2 M	2/2	NA		2/2	-	Case 2: multiple SEN and a large tumor occupying the frontal, temporal, and parietal lobes and compressing the lateral ventricles
TOTAL **n=36 studies**	**Total (n=387)** **S (n=118)** **M (n=269)**	**144/387 (37,2%)** **23/103 (22,3%)** **116/234 (49,6%)**	**163/257 (63,4%)** **27/76 (35,5%)** **136/181 (75,1%)**	**181/257 (70,4%)** **30/76 (39,5%)** **151/181 (83,4%)**	**239/387 (61,8%)** **38/118 (32,2%)** **201/269 (74,7%)**	**9/69 (13,0%)** **0/12 (0,0%)** **9/57 (15,8%)**	

CR, cardiac rhabdomyoma; CT, cortical tuber; DD, definite diagnosis; M, multiple; NA, not applicable; NVI, no variant identified; P/LP, pathogenic / likely pathogenic; S, single; SEN, subependymal nodule; VUS, variant of unknown significance

* Effective: number of positive clinical diagnoses with genetic testing

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MILON Vincent

Diagnostic de la sclérose tubéreuse de Bourneville en période prénatale : une étude rétrospective de 240 cas et revue de la littérature

RÉSUMÉ

La sclérose tubéreuse de Bourneville (STB) est une maladie rare multisystémique causée par un variant pathogène dans le gène *TSC1* ou *TSC2*. Une grande variabilité phénotypique caractérise la STB. Cette maladie prédispose à la formation d'hamartomes dans différents tissus, à des troubles neurologiques et neurodéveloppementaux tels que l'épilepsie, des troubles psychiatriques, et une déficience intellectuelle dans 50% des cas. La STB peut être à l'origine de rhabdomyomes cardiaques (RC), de tubers corticaux, ou de nodules sous-épendymaires durant la vie fœtale. La détection de RC multiples est associée à un très haut risque de STB, mais le RC peut être unique et isolé lors de sa détection. Peu de données existent pour estimer le risque de STB dans ces cas. Nous rapportons la plus grande série de tests génétiques prénataux pour la STB avec une étude rétrospective de 240 fœtus présentant des signes anténataux évocateurs. De plus, nous fournissons une revue de la littérature pour préciser la probabilité de diagnostic clinique ou génétique de STB en cas de détection de RC unique ou multiples. En effet, un diagnostic précoce est crucial pour le conseil des couples et de leurs familles. Dans cette série, un diagnostic définitif de STB a été porté chez 50% (41/82) des fœtus qui présentaient initialement un CR unique et 80,3% (127/158) en cas de RC multiples. Nous avons détecté 3 cas de mosaïcisme

Mots-clés : Sclérose tubéreuse de Bourneville ; STB ; Diagnostic prénatal ; Rhabdomyome cardiaque ; Tuber cortical ; Nodule sous-épendymaire

Diagnosis of tuberous sclerosis in the prenatal period: a retrospective study of 240 cases and review of the literature

ABSTRACT

Tuberous sclerosis complex (TSC) is a rare multisystemic disorder caused by a pathogenic variant in the *TSC1* or *TSC2* gene. A great phenotypic variability characterizes TSC. The condition predisposes to the formation of hamartomas in various tissues, neurologic and neurodevelopmental disorders such as epilepsy, psychiatric disorders, as well as intellectual disability in 50%. TSC may be responsible for cardiac rhabdomyomas (CRs), cortical tubers, or subependymal nodules during fetal life. Detecting multiple CRs is associated with a very high risk of TSC, but the CR could be unique and isolated when detected. Few data exist to estimate the risk of TSC in these cases. We report the largest series of prenatal genetic tests for TSC with a retrospective study of 240 fetuses presenting with suggestive antenatal signs. We also provide a review of the literature to specify the probability of clinical or genetic diagnosis of TSC in case of detection of unique or multiple CRs. Indeed, an early diagnosis is crucial for the counseling of the couple and their families. In series, a definite diagnosis was assessed in 50% (41/82) of fetuses who initially presented with a unique CR and 80.3% (127/158) in case of multiple CRs. We detected 3 cases of parental germinal mosaicism and estimated the risk at 2.6% (3/115).

Keywords : Tuberous Sclerosis Complex; TSC; Prenatal diagnosis; Cardiac Rhabdomyoma; Cortical tuber; Subependymal nodule

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