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Prenatal phenotype of 22q11 micro-duplications: a systematic review and report on 12 new cases

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Author statement

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Abstract

The 22q11 region is prone to generating recurring Copy Number Variations (CNVs) as a result of the large numbers of Low Copy Repeats (LCRs). Typical duplications encompass the LCR-A-to-D region but atypical duplications of various sizes have also been reported. These duplications are responsible for highly variable phenotypes with incomplete penetrance and expressivity, which is challenging for adequate genetic counselling, especially in the prenatal period. To better delineate prenatal phenotypes associated with these CNVs, we report here a clinical and molecular description of twelve cases (9 foetuses and 3 deceased new-borns babies) carrying recurrent 22q11 duplications (diagnosed via aCGH), along with a review of the existing literature. 22q11 duplications were inherited from an apparently healthy parent in almost 60% of the cases. Other CNVs were diagnosed for 8% of the cases. Increased nuchal translucency and cardiac anomalies (CHD) were the most prominent phenotypes observed, along with mild renal and skeletal anomalies. Duplications encompassing the LCR-C-to-D region (and the *CRKL* gene) seemed more likely to generate CHDs and renal malformations. Cleft lip/palate were observed in foetuses with duplications encompassing the LCR-A-to-B region or the *SPECC1L* gene, as previously suggested. However, genotype-phenotype correlations remain difficult to ascertain. Second-hit point variants, epigenetic or environmental variations could play a role in the phenotypic variability of 22q11 duplications, but remain a challenge for assessment in the short period of pregnancy.

Keywords: prenatal – 22q11 – aCGH – genotype-phenotype – foetal

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Introduction

Among pregnancies suspected to be at risk of a chromosomal anomaly (because of advanced maternal age or abnormal first trimester screening), an abnormal foetal karyotype is detected in 3.6% of the cases. The diagnostic rate can reach 35% in case of anomalies detected by ultrasound. The use of chromosomal microarrays (CMAs, i.e. aCGH and SNP-array) in prenatal routine diagnoses increases the diagnostic rate for foetal chromosomal abnormalities to between 3.6% and 5.2% depending on the indication (Lichtenbelt *et al.* 2011). Since the detection threshold for CMA is between 1 kb (for some types of SNP-array) and 1Mb (for aCGH using BACs), only very small imbalances (under the CMA detection threshold, such as point mutations and indels), uniparental disomy, low levels of mosaicism and balanced anomalies can be missed using these techniques. The precision of CMA is counterbalanced by difficulties in classifying some CNVs for the foetal phenotype. Tonni *et al.* (Tonni *et al.* 2019) demonstrated that in almost 20% of the cases, one of the parents was also a carrier of a CNV that was assumed to be causative for their foetus. Variants of unknown significance (VOUS) and CNV with reduced penetrance and variable expression raise the issue of the role of such variations in the foetal phenotype for which counselling should be provided for the parents (Lichtenbelt *et al.* 2011).

Chromosome 22 is prone to non-allelic homologous recombination (NAHR) due to the large numbers of lowcopy repeat sequences (LCR, namely LCR-A to H in the 22q11.2 region). This mechanism generates CNVs (duplications and deletions) with recurrent breakpoints, found among patients presenting various phenotypes. The most frequent CNV in the 22q11.2 region is a recurrent deletion between LCR-A and D, leading to the 22q11.2 deletion syndrome. Various duplications have also been described in the 22q11.2 region. Duplications encompassing the LCR-A to D region have been found to be responsible for mild neurodevelopmental phenotypes to severe congenital anomalies, such as bladder extrophy or cardiac malformations (de La Rochebrochard *et al.* 2006; Draaken *et al.* 2010). Duplications encompassing other LCRs are rarer. In most cases (60 to 80%), these duplications are inherited from an apparently healthy parent. This variability in penetrance

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and expressivity is a challenge when in-depth genetic counselling is required. The increased use of CMAs in the prenatal period in case of an abnormal ultrasound (US) examination also increases the diagnosis of 22q11.2 duplications among foetuses. However, descriptions of prenatal phenotypes associated with these duplications are rare. In an attempt to better delineate prenatal phenotypes associated with these variations, we provide here a clinical and molecular description of 9 foetuses and 3 deceased new-born babies that were carriers of recurrent 22q11.2 duplications, along with a review of the existing literature focusing on the prenatal period.

Patients and Methods

Patients

Of the 12 patients, 9 were foetuses referred for abnormal ultrasound screening results and 3 were new-born babies who died shortly after birth (of whom 2 had abnormal prenatal ultrasound results). All the patients in this study will be referred to hereafter by the term "foetus," to avoid confusion with data from the literature. Among the 9 pregnancies, two women had chosen to terminate the pregnancy (termination of pregnancy, TOP) following a cytogenetic diagnosis, 2 foetuses died *in utero* and 5 pregnancies led to a live birth. Standardized autopsy procedures were performed on all deceased foetuses or new-born babies, including skeletal X-rays, macroscopic examinations and histology analyses.

For all the patients, written informed consent for genetic testing and possible publication of this anonymized data were obtained from their legal representative(s).

CMA (Chromosomal MicroArray) and Fluorescence In Situ Hybridization (FISH) analysis

In brief, for all foetal samples, oligonucleotide aCGH was performed using the Agilent Human Genome CGH microarray 180 K, with 13 kb genome-wide median probe spacing (Agilent Technologies, Santa Clara, CA, USA). The experiments were performed according to the manufacturer's protocol. Patients' gDNAs were obtained from total blood (for parents), and from chorionic villi, amniotic fluid or frozen tissues (for the foetuses) using a Prepito instrument

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(PerkinElmer Inc., Turku, Finland). Images were analysed using Agilent Feature Extraction Software version 10.7.3.1. A graphic overview and analysis of the data were obtained using Agilent CytoGenomics software version 3.0.6.6. The probes were mapped using the GRCh37 genome version. Aberrant signals including five or more adjacent probes were considered as genomic aberrations and were retained for further study. FISH analysis, using the Vysis LSI TUPLE / LSI ARSA Probe Set (Abbott Laboratories. Abbott Park, Illinois, U.S.A), was performed to confirm the 22q11.2 typical duplication in the propositus and to study the possibility of inheritance when parental samples were available. In-house BAC probes were used to study atypical 22q11 duplications that were not targeted by the TUPLE1 probe, along with other CNVs when necessary, according to the CMA results. A final classification of the variants was based on data found in the literature and international CNV databases (DECIPHER, DGV, gnomAD), and on inheritance and gene content of the CNV, in accordance with recommendations by the French aCGH network (AChro-Puce).

Exome sequencing

HTS libraries were prepared with SureSelect XT2 Human All Exon V7 capture reaction (Agilent, Santa Clara, California, USA) according to the manufacturer's protocol. Paired-end sequencing (2×150-bp) was performed on an Illumina NextSeq 550. Variants detected, short indels and single nucleotide variants (SNVs), were annotated and ranked using C-GeVarA (UF Bioinformatique et Génétique Computationnelle, CHU Rennes).

Results

Cytogenetic studies

Twelve foetuses carrying duplications at the 22q11 locus, were included in this work. Most of these duplications were inherited from an apparently healthy parent (Table 1, Table 2 and Supplementary Table 2). Six duplications were typical (between LCR-A and LCR-D, foetuses F1 to F6, Figure 1) and 6 were considered as atypical duplications (foetuses F7 to F12, Figure 1). All breakpoints of atypical duplications took place in the LCR-rich sequences. A second CNV considered as

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pathogenic or VOUS was diagnosed for 3 foetuses (foetuses F9, F10 and F12, Supplementary Table 2). All the clinical and cytogenetic data for the 12 foetuses reported here can be found in the DECIPHER database at the following reference numbers: 433453 (F1), 433454 (F2), 433455 (F3), 433456 (F4), 433457 (F5), 433459 (F6), 433441 (F7), 433443 (F8), 433446 (F9), 433450 (F10), 433451 (F11), 433452 (F12).

Clinical description

Typical duplication carriers

The male-to-female ratio was 1.5. The mean term at diagnosis was 23.2 weeks gestation (WG). In half of the cases, the duplication was inherited from an apparently healthy parent. No other pathogenic CNV was found. Detailed clinical information on each foetus is summarized in Table 1 and Supplementary Table 1. The most common clinical findings were increased nuchal translucency (or hygroma colli) in 50% of the cases and cardiac anomalies in 80% of the foetuses. Most of these anomalies were valvular or great vessels malformations (4 foetuses). Two foetuses (F1 and F2, 33%) presented growth anomalies. Miscellaneous findings were observed for three of the foetuses: microcephaly with short corpus callosum (F6), brachymesophalangy (F1), and facial gestalt (F1). No renal, genital-urinary, palatal or skeletal malformations were observed.

Literature review

Thirty-seven patients, all carriers of 22q11.2 typical duplication with prenatal data, had previously been reported (Cai *et al* 2020; Christopoulou *et al.* 2013; de La Rochebrochard *et al.* 2006; Dupont *et* al. 2015; Hu *et al.* 2011; Schramm et al. 2011; Tonni *et al.* 2019; Yu *et al.* 2019). The male-to-female ratio was 1 to 1. The mean term at diagnosis was 20.5 WG. Detailed clinical information on each foetus is summarized in Table 1 and Supplementary Table 1. In 63% of the cases, the duplication was inherited from a healthy parent. No other pathogenic CNVs were reported, except for one foetus presenting homogeneous X monosomy. Termination of Pregnancy (TOP) was more frequently requested in previously reported cases than in our cohort (46% vs. 17%). Typical duplication carriers were mainly referred for increased nuchal translucency (32%) and

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cardiovascular anomalies (33%). Cardiovascular malformations ranged from a single umbilical artery to more complex phenotypes with valvular thickening, stenosis or atresia associated with left ventricle hypertrophy or septal defects (Supplementary Table 1). Five foetuses presented facial anomalies, ranging from dysplastic ear to cleft palate and micrognathia. Three foetuses presented growth anomalies. Thymic hypoplasia was observed in two of them. Reported skeletal anomalies were rare and mild (11%). One foetus presented caudal regression syndrome associated with a clubfoot. Miscellaneous findings (involving one patient each) included thoracic heterotaxia and abdominal situs inversus, imperforate anus, oesophageal atresia, a duplicate kidney with vesicoureteral reflux associated with hypospadias, and enlarged lateral ventricles.

Atypical duplication carriers

In our cohort, the male-to-female ratio was 1.5. Among the 6 carriers, TOP was requested only once (F11), 2 foetuses died intra-uterus (F7 and F10) and 3 carriers (F8, F9 and F12) died in early infancy (before 3 months,F8, F9 and F12). Three foetuses were carriers of a second CNV (F9, F10, F12, Table 2). The mean term at diagnosis was 15.3 WG. For F8 and F9 a postnatal molecular diagnosis was carried out .

Comparison with the phenotype of typical duplication carriers showed similar frequencies for increased nuchal translucency (50% in each group), cardiovascular anomalies (66% in atypical duplication carriers vs. 80%) and skeletal malformations (33% in atypical duplication carriers vs. 20%). Miscellaneous findings such as mild CNS or growth anomalies were also reported in this group. However, facial and renal malformations were more frequent among atypical duplication carriers (66% vs. 0%).

Thirteen foetuses or infants were reported in the literature (Cai *et al.* 2020; Cicatiello *et al.* 2019; Dupont *et al.* 2015; Li *et al.* 2019; Maya *et al.* 2017; Tonni *et al.* 2019; Yu *et al.* 2019). The male to female ratio was 2.5. Delivery of a living child was observed in 7 cases; TOP was performed in 4 cases, early infantile death was observed twice. In two cases, the outcome of the pregnancy was unknown. The mean term at diagnosis was 22.1 WG but 4 patients were diagnosed at birth or

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thereafter (Yu *et al.* 2019). Detailed clinical information on each patient is summarized in Table 2 and Supplementary Table 2.

Duplications between LCR-A and LCR-B (~918 kb)

These duplications were not found in our cohort but 2 fetuses or infants were reported in the literature ((Dupont *et al.* 2015), P3 and P4). One foetus was diagnosed prenatally because the father was known to carry the duplication, but no pathogenic US signs were identified and the child was born full term and healthy. The other pregnancy was interrupted at an unspecified term. The foetus presented cardiac anomalies and facial gestalt with a cleft palate and delayed bone maturation. In this family, the duplication was also inherited from the father. One additional foetus with a larger proximal duplication encompassing LCR-A and B was described by Cicatiello *et al.* 2019, who only reported increased nuchal translucency.

In one additional case, the size of the duplication was only 8.5 kb (Tonni *et al.* 2019) and thus did not encompass the region between LCR-A and LCR-B. The foetus also presented a ventricular septal defect and a transposition of the great vessels.

Finally, four other children carrying these duplications also presented a neurodevelopmental phenotype, growth delay and microcephaly without any other malformations (Ou *et al.* 2008).

Duplications between LCR-C and D (~388 to 472 kb)

Foetus F12 presented this duplication, which was inherited from the father. The father also transmitted a 1.55Mb 15q13.2q13.3 duplication to his foetus. This male foetus presented prenatal hydrops and died after 1 hour of life. Autopsy procedures showed facial gestalt, cryptorchidism and renal pelvic duplication. The additional 15q13.2q13.3 duplication mirrors the 15q13.3 deletion syndrome and thus prompted us to consider this CNV as a VOUS rather than a benign variant.

Three foetuses or new-born babies have been reported with a similar duplications in the literature ((Yu *et al.* 2019) case 5, (Li *et al.* 2019) patients 24 and 25). The foetus reported by Yu *et al.* 2019)presented a complex phenotype including shortened extremities with brachydactyly and polydactyly of the left foot, short ribs, abnormal mineralization of the cranium posterior fossa,

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cardiac anomalies and partial situs inversus. He died shortly after birth. One foetus presented isolated valvular anomalies, ((Li *et al.* 2019), patient 25). Finally, one foetus ((Li *et al.* 2019) patient 24) was screened because of advanced maternal age and carried a duplication inherited from the father. No other anomalies were observed. These two last foetuses were born full term.

One new-born baby was reported by (Ou et al., 2008) with isolated abnormal sexual development. His father, carrying the duplication, was asymptomatic.

Duplications between LCR-D and E (~1.2 Mb)

One foetus from our cohort carried this duplication (F11). The duplication was de novo. The foetus was diagnosed with increased nuchal translucency, facial gestalt, brachydactyly and renal hypertrophy. TOP was requested at 22 WG. No other foetuses have been reported in the literature. Ou (Ou *et al.* 2008) reported one child with developmental delay and facial gestalt, whose duplication was inherited from a healthy father.

Duplications between LCR-E and F (~656 kb)

Li *et al.* 2019 reported a patient with this duplication. The diagnosis was made after an invasive sampling for echogenic bowel. The couple chose to pursue the pregnancy and the patient gave birth to a healthy girl.

Another child was reported postnatally by Sedghi *et al*. 2015: the girl presented developmental delay and cleft palate. She inherited the duplication from her healthy mother.

Duplications between LCR-F and H (~1.30 Mb to 1.35 Mb)

Foetuses F7, F9 and patient 26 (Li *et al.* 2019) carried duplications between LCR-F and LCR-H. In one foetus, the duplication was maternally inherited (F9). For F7, the mother was not a carrier but the father could not be tested. Foetus F7 died in utero at 10 WG in a context of increased nuchal translucency followed by hydrops. On autopsy she presented Fallot tetralogy, low-set ears and a posterior palatine cleft. F9 was diagnosed at 2 months of age because of sudden infant death syndrome, which triggered genetic analyses. Prenatal ultrasound examinations were reported as normal. Autopsy procedures diagnosed thymic agenesis, bifid uvula and retroesophageal right

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subclavian artery. The male patient was also a carrier of a duplication of the *MID1* first exon (Xp22.2) first considered as a VOUS and then later classified as likely to be benign since it had been inherited from his healthy maternal grandfather. Patient 26 (Li *et al.* 2019) was diagnosed after a positive second-trimester screening without any anomalies on ultrasound. The duplication was inherited from the mother.

Five children or adults were reported (Chang *et al.* 2015; Tan *et al.* 2011) with similar duplications. Most of these patients presented a different phenotype including developmental delay, hypotonia and cerebral malformations, without any other organ involvement.

Larger duplications: duplications between LCR-A and LCR-C (~2.4 Mb)

One foetus with this duplication was reported in the literature ((Yu *et al.* 2019), case 6) of unknown inheritance. The foetus was also a carrier of trisomy 18.

Larger duplications: duplications between LCR-B and LCR-D (~745 kb to 1.07 Mb)

One male new-born baby (F8, born preterm at 29 WG) was diagnosed prenatally with hydrops fetalis, mild ventricular dilatation, a remnant of the left umbilical vein and pyelo-ureteral duplication. He died at day 2 from pulmonary hypoplasia and delayed lung maturation. Autopsy procedures confirmed the ultrasound anomalies and highlighted facial gestalt on an overgrown male baby. His older female sibling had died at day 20 (after a preterm birth at 28 WG) with pulmonary hypertension and pleural effusions. The female sibling also presented periventricular leukomalacia and pyelo-ureteral duplication and was a carrier of the atypical duplication inherited from their healthy father. Owing to the recurrence of this severe phenotype with a poor outcome, quartet exome sequencing was performed and no additional pathogenic variation was found. Twins were reported with a similar duplication in the literature (Cai *et al.* 2020). They were diagnosed with isolated polycystic kidneys. The duplication occurred de novo and the twins were born full term.

Larger duplications: duplications between LCR-D and LCR-F (~1.94 Mb)

Maya (Maya *et al.* 2017) reported a foetus carrying a duplication between LCR-D and F, who

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presented a hypoplastic right kidney, an aberrant right subclavian artery and choroid plexus cysts. The inheritance of the duplication and the pregnancy outcome were unknown.

Ou (Ou *et al.* 2008) also reported a new-born baby with multiple congenital anomalies including imperforate anus, kidney and skeletal anomalies, patent ductus arteriosus, patent foramen ovale, and an abnormal right subclavian artery.

Larger duplications: duplications between LCR-E and LCR-H (~1.81 Mb)

Foetus F10 inherited this duplication from her father. She was also a carrier of a chromosome-18 derivative of a paternal t(8;10)(q22.1;q26.11), leading to a 10q26.11q26.3 duplication (15,9Mb) and a 18q22.1q23 deletion (11,6Mb). The diagnosis was made at 14 WG following intrauterine death. The chromosome 18 derivative was probably responsible for the intrauterine death and some of the phenotypes observed. No other prenatal cases have been reported previously.

Zhang (Zhang *et al.* 2021) reported one Chinese patient with this duplication who was referred for neurodevelopmental anomalies and congenital heart defect.

Discussion

The chromosome 22q region is prone to CNVs by NAHR and mediated by a complex set of segmental duplications. Deletions and duplications are responsible for various phenotypes, ranging from asymptomatic carriers to severe multi-organ malformations (de La Rochebrochard *et al.* 2006; Draaken et al., 2010; Ou *et al.* 2008). This great variability raises problems of interpretation when 22q11 duplications are diagnosed in the prenatal period. It can be difficult to link a prenatal phenotype to these duplications, since only a few previous reports have focused on descriptions of the carriers' prenatal phenotypes. In this study, we focused on the prenatal phenotype of 22q11 duplications, reporting 12 novel foetuses and a review of the literature.

Prenatal phenotype of 22q11 duplication carriers and phenotype-genotype correlations

Non-specific phenotypes and asymptomatic carriers

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Increased nuchal translucency was one of the most frequent indications for invasive sampling in this cohort (31%). Moreover, when compiling data from this cohort and the literature, 14% of the foetuses carrying an atypical duplication (and 19% of those with typical duplication) did not appear to present any abnormal ultrasound results: the duplication was an incidental finding after analyses were performed for positive Down's syndrome screening or advanced maternal age.

Cardiac anomalies

Cardiovascular malformations were a prominent anomaly among the foetuses we reported (40%), including atrial and/or ventricular septal defects, Fallot tetralogy and valvular anomalies. Many genes have been suspected to be responsible for the CHD observed among 22q11.2 CNV carriers (*CRKL, TBX1, MAPK1, HIRA, CECR1, DGCR6, DGCR8, HIC2* and *PRODH*, (Carli *et al.* 2021; Morrow *et al.* 2018)) and *HIRA, TBX1* and *CECR1* were incriminated among CHD patients who were carriers of 22q11 duplications of various sizes in both the prenatal and postnatal periods (Carli *et al.* 2021). Except for *CECR1* and *MAPK1*, all of these genes are located in the typical 22q11.2 interval. Narrowing the interval has led to conflicting results in the literature between LCR-A to LCR-B (containing *HIRA* and *TBX1*) and LCR-C to LCR-D (containing *CRKL*)(Zhao et al., 2020). In our study, the region between LCR-C and LCR-D (containing *CRKL*) seemed more prone to generate CHD among duplication carriers (Supplementary Figure 1), but 4 CHD foetuses carried duplications distal to LCR-D. Recent work by Zhao *et al.* 2020 suggested that the presence of rare SNPs in a regulatory region of the *CRLK* gene in the normal 22q11 region among hemizygous patients could increase the risk of CHD by influencing the level of *CRKL* expression. In fact, *CRKL* is a dose-sensitive gene, and mouse models combining various hypomorphic alleles of this gene presented CHD (Racedo *et al.* 2015). We can postulate that these SNPs could also play a role in the phenotypical variability observed in duplication carriers by modifying *CRKL* levels of expression, either in foetuses with duplications not containing this gene (with a negative impact of one hypomorphic *CRKL* allele), or in foetuses with duplications containing *CRKL*, if they only carry hypomorphic *CRKL* alleles on both chromosomes 22. However, this hypothesis needs further

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exploration.

Skeletal anomalies

Various mild skeletal anomalies have been observed in up to 17% of foetuses (this study, Dupont *et al.* 2015; Yu *et al*. 2019), Supplementary Figure 2), mainly brachydactyly, polydactyly and crisped hands. Anomalies close to those observed in this cohort were also reported among carriers of LCR-D to LCR-E/F deletions (Ben-Shachar *et al.* 2008).

Renal and urinary anomalies (CAKUT)

Moderate renal anomalies were reported for 15% of the foetuses reported here. Although these anomalies are close to those reported among 22q11.2 deletion carriers, not all the foetuses carried duplications overlapping the critical deleted region for kidney malformations (between LCR-C and LCR-D, (Lopez-Rivera *et al.* 2017, p.), Supplementary Figure 4). In this interval, *CRKL* has been suggested as a critical mediator of developmental processes of the kidney (Hall *et al.* 2017). More recently, *CRKL* has also been linked to bladder extrophy, in addition to *THAP7*, and *LZTR1* among 22q11.2 duplication carriers (Draaken *et al.* 2010), a phenotype we did not find among the foetuses reported here.

Facial anomalies

Cleft lip and/or palate were reported for 10% of the foetuses. The 22q11 region is suspected to be a risk factor for ocular-auricular-vertebral spectrum malformations (including micrognathia, dysplastic ears and cleft lip/palate, (Glaeser *et al.* 2020). Four of the six foetuses with cleft lip/palate carried duplications including the LCR-A to LCR-B interval (Supplementary Figure 3). Other candidate genes include *CLTCL1, GSC2, HIRA, MAPK1, TBX1,* and *YPEL1* (Glaeser *et al.* 2020). Functional studies, where *SPECC1L* expression in mice had been reduced, showed that this dose-sensitive gene regulated palate development (Hall *et al.* 2020). This gene is located in the region duplicated in the two foetuses with cleft lip/palate (F7 and F10, Supplementary Figure 3) but it is worth noting that the chromosome-18 derivative observed in F10 could also have played a role in the occurrence of the facial anomalies observed.

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Thymus agenesis and hypoplasia

Thymus hypoplasia or agenesis was reported in 5% of the prenatal cases reported here (F9, (Dupont *et al.* 2015)). *TBX1* has been implicated in these anomalies through its preeminent role in the development of the pharyngeal apparatus (Gao *et al.* 2013). In mouse models, either overexpression or *TBX1 KO* leads to abnormal thymus development (Gao *et al.* 2013). However, foetus F9's duplication was distal to *TBX1*, suggesting that other mechanisms modifying thymus establishment and growth, such as potential abnormal *TBX1* expression through a disruption in topologically-associated DNA domains (TAD).

Shedding light on phenotypical variability

Despite the fact that we reported a large cohort of prenatal data on foetuses with various duplication sizes, this study did not allow specific phenotypes to be linked to particular duplicated regions at the 22q11 locus. We were not able to fully confirm the implication of some critical regions suggested in previous studies (see above). Moreover, considerable intra-familial variability was noted, with inherited duplications observed in both asymptomatic foetuses and neonatal deaths. However, study of the relatives is necessary for in-depth genetic counselling. Both CMA and FISH analyses can be used for this purpose. Although duplications can be challenging to diagnose using FISH, we preferred this technique to CMA. First, targeted studies of this sort enable the localization of the duplicated segment on metaphases. Even if NAHR is prone to generating tandem duplications, rarer anomalies can be dismissed using FISH (inserted duplication, cryptic translocation etc.). Moreover, FISH performs better than CMA in detecting low rates of mosaicism (Mary *et al.* 2021), which is of importance for genetic counselling. Finally, FISH is a targeted analysis and avoids incidental, non-desirable genomic imbalance findings among apparently healthy parents.

Second-hit CNV and point variations

For some of our subjects (and for previously reported subjects, such as P10 (Dupont *et al.* 2015), and case 6 (Yu *et al.* 2019)), aneuploidy or second-site CNV explained most of the symptoms. However, the most severely affected foetuses reported here were not always carriers of second-site

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CNV explaining their phenotype. Additional point variations cannot be excluded (except for F8). In fact, recent studies have warned against attributing a severe phenotype to variably penetrant CNVs by demonstrating the co-occurrence of additive pathogenic point variations among severely affected patients (Demily *et al.* 2018), or the additional burden of rare SNPs (Zhao *et al.* 2020). These variations of a single base are beyond the diagnostic capacities of CMA, and nowadays exome sequencing is suggested as a second-tier routine examination when faced with ultrasound anomalies, if CMA is considered normal. In the prenatal period, exome sequencing can increase the CMA diagnostic rate, but the overall detection of causative genetic variants in exome sequencing is lower than that suggested by previous smaller-scale studies (Lord *et al.* 2019). In our study, exome sequencing performed on one foetus and his sibling, both carriers of the atypical 22q11 duplication and both presenting the same clinical features, did not highlight any additional pathogenic variation, and whole genome sequencing still remains a challenge during the short period of pregnancy .

Stochastic events and epigenetic variations

It has been demonstrated that even homozygous twins carrying 22q11.2 deletions have discordant phenotypes (Morrow *et al.* 2018), suggesting environmental or epigenetic modifiers. In fact, numerous long non-coding RNAs and at least two small nucleolar RNAs (snoRNAs) are located in the 22q11.2 region, and in a recent review by it was suggested that the role played by copy number modification of these RNAs was one explanation for phenotypical variability, at least in 22q11.2 deletions. The LCR-A to LCR-D interval also contains *DGCR8*, a gene coding for a nuclear microRNA binding protein required for the biogenesis of microRNAs: changes in the levels of expression of this protein could lead to a hyper-variable pattern of hundreds of microRNAs from one patient to another (Du *et al.* 2019). Finally, a recent study on schizophrenic 22q11.2 deletion carriers suggested that methylation alterations in specific imprinting genes outside chromosome 22 could be a burden for the neuropsychiatric phenotype of these patients. These hypotheses are yet to be explored among 22q11 duplication carriers.

Conclusion

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22q11.2 recurrent duplications are challenging when diagnosed during pregnancy. Their high variability in penetrance and expressivity make genetic counselling difficult. In this work, involving one of the largest cohorts of prenatal 22q11.2 duplication carriers, we highlighted that increased nuchal translucency and cardiac malformations were frequently observed among these patients. However, the various foetal malformations reported here suggest that this duplication alone cannot explain the whole phenotype. A second genetic hit, a burden of rare variants or other extrinsic factors could explain this clinical variability. Exome sequencing may be of interest in case of absence of second-site pathogenic CNVs on prenatal CMAs and, in the near future, whole genome sequencing could help to detect both SNP and CNV in one technique, but applications of these techniques in the duration of pregnancy could be limited or challenging. However, exome or genome sequencing could be of great interest in the post-natal period, whatever the outcome of the pregnancy, to provide parents with the most accurate genetic counselling.

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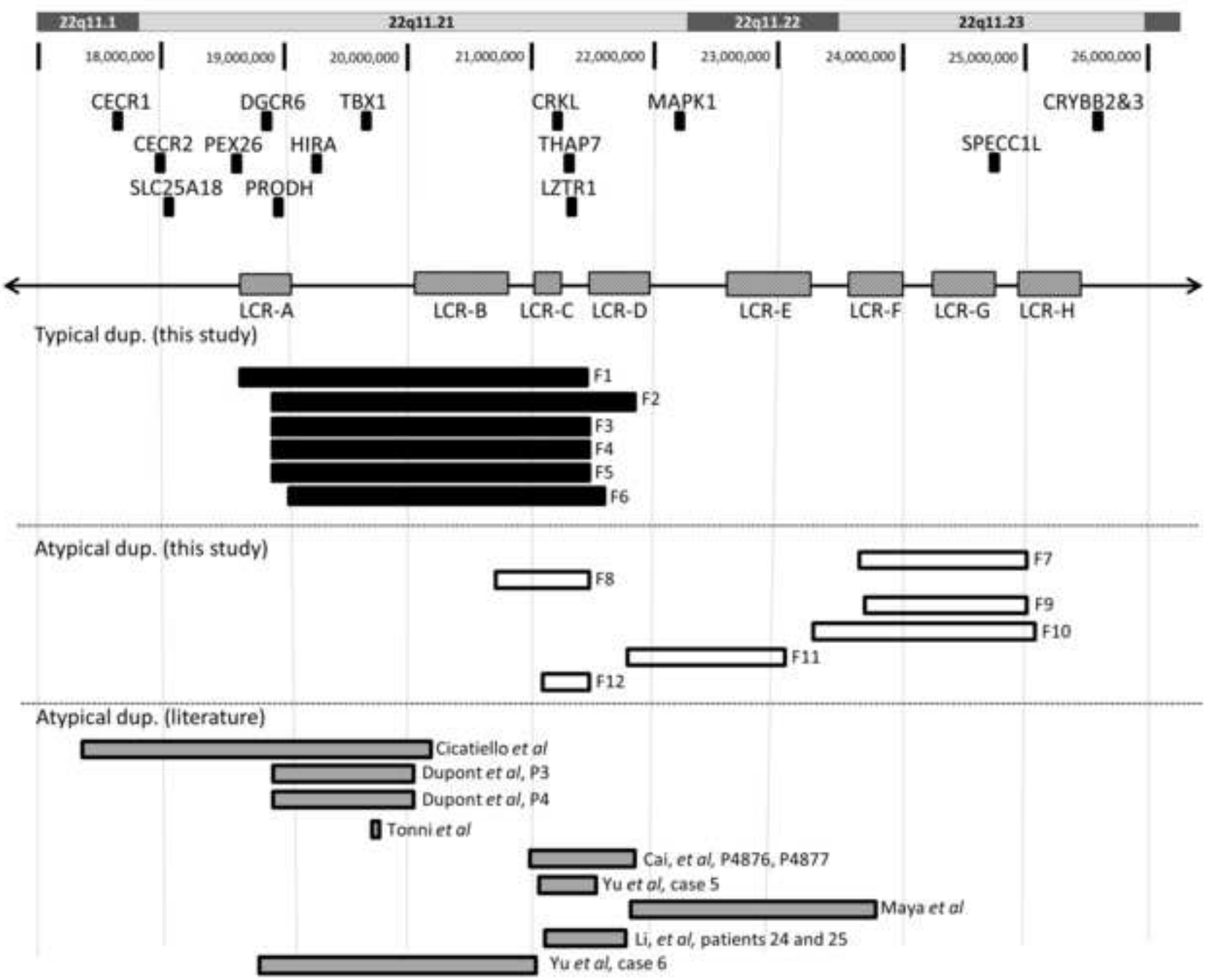
Figure Titles and Legends:

Figure 1: Graphic representation of the 22q11.2 duplications reported in this work and in the literature (adapted from the UCSC Genome Browser, zoom between chromosome 22 position 17.000.000 and position 26.000.000, hg19). Black and white boxes represent the foetuses described in this work; light grey boxes represent the duplications previously reported. Small black squares identify the approximate positions of the genes of clinical interest. Grey boxes represent the chromosomal bands. Dup.: duplication.

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Figure

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Number of foetuses	*This study (n=6)*	*Literature (n=37)*
Inheritance	De novo (2) Mother (1) Father (2) Unknown (1)	De novo (10) Mother (9) Father (8) Unknown (10)
Outcome	Live birth 5/6 TOP 1/6	Live birth 16/37 TOP 17/37 On-going pregnancy 4/37
Clinical presentation		
Increased nuchal translucency	3/6	12/37
Growth anomalies	Overgrowth (1) IUGR (1)	Overgrowth (1) IUGR (2)
Microcephaly	1/6	1/35 Unknown (1/37)
Other CNS anomalies	1/6	2/16 Unknown (21/37)
Facial gestalt	1/6	3/24 Unknown (13/37)
Facial anomalies	0/4 Unknown (2/6)	5/36 Unknown (1/37)
Cardiovascular anomalies	4/5 Unknown (1/6)	12/36 Unknown (1/37)
Skeletal anomalies	1/5 Unknown (1/6)	4/36 Unknown (1/37)
Genito-urinary anomalies	0/5 Unknown (1/6)	1/36 Unknown (1/37)
Renal anomalies	0/6	2/36 Unknown (1/37)
Other symptoms	0/6	12/37

Table 1: Clinical summary of foetuses carriers of typical 22q11.2 duplications.

TOP: Termination Of Pregnancy, IUGR: Intra-Uterine Growth Retardation, CNS: Central Nervous System

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LCR interval	*LCR-A to LCR-B*	*LCR-C to LCR-D*	*LCR-D to LCR-E*	*LCR-E to LCR-F*	*LCR-F to LCR-H*	*LCR-A to LCR-C*	*LCR-B to LCR-D*	*LCR-D to LCR-F*	*LCR-E to LCR-H*
Inheritance	Father (2)	Father (2) Mother (1) Unknown (1)	De novo	Unknown	Mother (2) Unknown (1)	Unknown	Father (1) De novo (2)	Unknown	Father (1)
Outcome	TOP (1) Living birth (1)	Early death (2) Living birth (2)	TOP	Living birth	IUD (1) Early death (1) Living birth (1)	Early death	Early death (1) Living birth (2)	Unknown	IUD (1)
Number of foetuses (this study & literature)	4	4	1	1	3	1	3	1	1
Clinical presentation									
Increased nuchal translu-cency	1/4	1/4	1	0	1/3	0	0/5	0	0
Growth anomalies	0/4	0/4	0	0	0/3	1	1/5	0	0
Microcephaly	0/4	0/4	0	0	0/3	1	0/5	0	0
Other CNS anomalies	0/4	0/4	0	0	0/3	1	0/5	1	0
Facial gestalt	1/4	1/4	1	0	1/3	0	1/5	0	1
Facial anomalies	0/4	0/4	0	0	1/3	1	0/5	0	1
Cardiovascular anomalies	2/4	2/4	0	0	2/3	1	1/5	1	1
Skeletal anomalies	1/4	1/4	1	0	0/3	1	0/5	0	1
Genito-urinary anomalies	0/4	1/4	0	0	0/3	0	0/5	0	0
Renal anomalies	0/4	1/4	1	0	0/3	0	3/5	1	1
Other symptoms	0/4	1/4	0	1	1/3	1	1/5	0	0

Table 2: Clinical summary of foetuses carriers of atypical 22q11 duplications.

TOP: Termination Of Pregnancy. IUD: Intra-Uterine Death. CNS: Central Nervous System.

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Supplementary data, Titles and Legends:

Supplementary Table 1: Detailed cytogenetics and clinical data for foetuses carriers of typical 22q11.2 duplications.

VOUS: Variant Of Unknown Significance, WG: Gestation Weeks, TOP: Termination of Pregnancy, IUGR: Intra-Uterine Growth Retardation, IUD: Intra-uterine death, mat: maternal, pat: paternal, CNS: Central Nervous System, NC: not communicated, dX: X days of life, m-o: months-old, VSD: Ventricular Septal Defect, ASD: Atrial Septal Defect, ID: Intellectual Deficiency.

Supplementary Table 2: Detailed cytogenetics and clinical data for foetuses carriers of atypical 22q11 duplications.

VOUS: Variant Of Unknown Significance, WG: Gestation Weeks, TOP: Termination of Pregnancy, IUGR: Intra-Uterine Growth Retardation, IUD: Intra-uterine death, mat: maternal, pat: paternal, CNS: Central Nervous System, NC: not communicated, dX: X days of life, m-o: months-old, VSD: Ventricular Septal Defect, ASD: Atrial Septal Defect, ID: Intellectual Deficiency.

Supplementary Figure 1: Graphic representation of the 22q11.2 duplications observed among foetuses presenting congenital cardiac defects (adapted from UCSC Genome Browser, zoom between chromosome 22 position 17.000.000 and position 26.000.000, hg19). Black boxes represent “typical” duplications. White boxes represent the “atypical” duplication carriers we reported and light grey boxes represent the “atypical” duplications previously reported in the literature. Small black squares identify the approximate positions of genes of clinical interest. Grey boxes represent the chromosomal bands. Dup.: duplication.

Supplementary Figure 2: Graphic representation of the 22q11.2 duplications observed among foetuses presenting skeletal anomalies (adapted from UCSC Genome Browser, zoom between chromosome 22 position 17.000.000 and position 26.000.000, hg19). Black boxes represent “typical” duplications. White boxes represent the “atypical” duplication carriers we reported and light grey boxes represent the “atypical” duplications previously reported in the literature. Small black squares identify the approximate positions of genes of clinical interest. Grey boxes represent

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the chromosomal bands. Dup.: duplication.

Supplementary Figure 3: Graphic representation of the 22q11.2 duplications observed among foetuses presenting cleft lip/palate (adapted from UCSC Genome Browser, zoom between chromosome 22 position 17.000.000 and position 26.000.000, hg19). Black boxes represent "typical" duplications. White boxes represent the "atypical" duplication carriers we reported and light grey boxes represent the "atypical" duplications previously reported in the literature. Small black squares identify the approximate positions of genes of clinical interest. Grey boxes represent the chromosomal bands. Dup.: duplication.

Supplementary Figure 4: Graphic representation of the 22q11.2 duplications observed among foetuses presenting renal anomalies (adapted from UCSC Genome Browser, zoom between chromosome 22 position 17.000.000 and position 26.000.000, hg19). Black boxes represent "typical" duplications. White boxes represent the "atypical" duplication carriers we reported and light grey boxes represent the "atypical" duplications previously reported in the literature. Small black squares identify the approximate positions of genes of clinical interest. Grey boxes represent the chromosomal bands. Dup.: duplication.

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