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Prenatal Exome Sequencing in Central Nervous System Anomalies: diagnostic yield and challenges

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ABSTRACT

Trio-based prenatal exome sequencing (pES) showed a significant incremental diagnostic yield over karyotype and chromosomal microarray (CMA) in fetuses with structural anomalies. However, optimized indications, detection rates in different categories of fetal anomalies, and interpretation of variants pathogenicity are still under investigation.

The aim of this study was to assess the incremental diagnostic yield of trio-based pES after karyotype and CMA inconclusive result in Central Nervous System (CNS) anomalies.

Between January 2019 and December 2022, a cohort of 33 fetuses presenting apparently isolated or non-isolated CNS anomalies was enrolled. In all cases karyotype and CMA analyses were performed. In non-conclusive cases, pES was offered. Trio-based pES was performed in 15 cases on genomic DNA extracted from fetal samplings and parental leukocytes. Library preparation and targeted enrichment were performed using the Twist Human Core Exome Kit and sequenced on the Illumina NovaSeq 6000 platform. Then, a systematic review on the published cohorts of fetuses specifically selected for CNS anomalies was performed according to PRISMA guidelines, including n=12 papers. The incremental diagnostic yield of pES over CMA/karyotype was calculated for each study and pooled in a meta-analysis using a logistic random mixed-effect model.

In our cohort in 4/33 cases (12%) standard karyotype was conclusive. CMA was not diagnostic in any case. In 5/15 cases (33%), pES disclosed likely pathogenic (LP) or pathogenic (P) variants in *BICD2, NFIA, ARID1A, RPGRIP1L* and *ZIC2* genes fitting the fetal phenotypes. In 6/15 (40%) cases, multiple Variants of Uncertain Significance (VUSs) were detected. In one case both VUSs and LP variants were detected, partially related to the fetal phenotype. In three cases no variants were disclosed. Systematic literature review showed an incremental yield ranging from 19% to 57% in antenatal cohorts focused on CNS anomalies. The pooled incremental diagnostic yield estimate resulted 36% (95% C.I.: [28%;44%]) including all CNS anomalies, 22% (95% C.I.: [15%;31%] in apparently isolated CNS anomalies, 32% (95% C.I.: [22%;45%]) in CNS-only related anomalies (one or more) and 45% (95% C.I.: [37%;53%]) in non-isolated CNS anomalies (either ≥ 2 anomalies in CNS, or extra-CNS).

In conclusion, meta-analysis showed a substantial diagnostic improvement in performing genome wide sequencing analysis over standard and molecular cytogenetics, supporting the proposal of performing pES earlier in the diagnostic route of CNS anomalies.

1.INTRODUCTION

1.1 Central Nervous System Anomalies:

1.1.1 Epidemiology

Malformations of the central nervous system (CNS) occur in 1 to 2:1000 births, however the true incidence is likely much higher (1/100) considering late-manifesting CNS anomalies and newer imaging technologies (Leibovitz et al., 2022; Van den Veyver, 2019).

1.1.2 Embriology and Pathogenesis

The development of the human CNS is complex and governed by successive embryonic and fetal key processes: neural tube formation, separation of the telencephalic vesicle, neuronal and glial proliferation, neuronal migration, and cortical organization.

Abnormalities in one of these processes led to the pathogenesis of CNS malformations (Leibovitz et al., 2022), and may be caused by specific chromosomal or monogenic disorders (Scelsa, 2022). During the first six weeks of embryonic life, CNS development is governed by three sequential, overlapping phases: gastrulation (formation of the three main germ layers, ectoderm, mesoderm, and endoderm); dorsal induction (creation of the neural tube with three primitive vesicles: prosencephalon, mesencephalon, and rhombencephalon); and ventral induction or telencephalization (separation of two cerebral hemispheres and formation of optic vesicles, olfactory bulbs, and corresponding facial structures) (Ten Donkelaar & Van der Vliet, 2006). All these processes are closely controlled, genetically and epigenetically (Reichard & Zimmer-Bensch, 2021).

The impaired embryonic and fetal CNS developmental process can cause specific congenital CNS anomalies: abnormal dorsal induction leads to neural tube defects; disorders of ventral induction manifest with various types of holoprosencephaly; unbalanced neuronal proliferation and apoptosis causes microcephaly or megalencephaly; inadequate neuronal migration results in lissencephaly and heterotopia; and failed cortical organization presents as polymicrogyria or cortical dysplasia (Leibovitz et al., 2022).

In addition, proper brain function relies on finely regulated epigenetically neurodevelopmental processes, which are by far not fully understood. Transcriptional interconnected networks regulate cortical neurogenesis, neuronal identity, cellular migration, cortical positioning, and the formation of neuronal connections (Paolino et al., 2020; Reichard & Zimmer-Bensch, 2021).

1.1.3 Anatomy, etiology, and prognosis of fetal CNS anomalies

1.1.3.1 Anomalies of Dorsal Induction: Neural Tube Defects

Neural tube defects (NTD) are caused by abnormal embryonic dorsal induction, which regulates formation and closure of the neural tube between the 5th and 7th postmenstrual weeks. NTDs are common CNS abnormalities that affect 0.5–2% of pregnancies.

The NTD spectrum includes anencephaly, cephalocele, and spina bifida (Leibovitz et al., 2022). According to the multisite closure model, fusion of the neural fold occurs at multiple locations along the developing neural tube and specific NTD types can be explained by failed fusion of the neural tube at the corresponding closure sites (Van Allen, 1996). Cranial NTD may present with a total absence of the cranial vault (acrania) or a partial skull defect with herniated intracranial contents (cephalocele). The incidence of anencephaly is about 1 in 1000 births and fetuses with acrania are frequently diagnosed at late first or early second trimester with exencephaly (*i.e.* cerebrum noncovered by the cranial vault).

The acrania-exencephaly-anencephaly sequence is the earliest and most severe destructive brain insult that occurs during gestation (Chaudhari & Ho, 2022). Exencephaly represents the intermediate stage with partial degeneration of cerebral hemispheres, while anencephaly represents the end stage of degeneration into angiomatous stroma with residual facial structures (Avagliano et al., 2018). Cranial meningocele is defined as the protrusion of meninges and cerebrospinal fluid trough a skull defect without neural tissue, while in encephalocele, cerebral tissue is also herniated. The occipital cephalocele is the most common subtype in Europe and the USA, while the frontal one is more common in Southeast Asia (Leibovitz et al., 2022). Fetuses with cephalocele are at increased risk of chromosomal anomalies and syndromic conditions, and two/third of cases may have additional CNS malformations.

Spina bifida refers to a defect in fusion of the spinal portion of the neural tube, affecting 1 in 1000 newborns and is classified as open or closed according to the absence or presence of skin tissue covering the defect, respectively. The mildest form is named spina bifida occulta, in which only the outer part of the affected vertebrae is not fused. The commonest form of spina bifida (95%) is open, manifesting as myelomeningocele (the spinal cord and/or nerves extend into the lesion) or meningocele (cystic buldge without neural tissues) with a skin defect above the lesion (Avagliano et al., 2018). Open NTD has multifactorial etiology. Most cases of spina bifida are of non-genetic origin, such as teratogenic causes including intake of antiepileptic drugs (*i.e.* valproic acid) and uncontrolled pregestational maternal diabetes mellitus (Chen, 2008). Periconceptional folic acid treatment can reduce NTD incidence by 60–70%, nevertheless, *MTHFR* gene mutations, which affect folate

metabolism, have not been shown to contribute significantly to NTD prevalence (Mohd-Zin, 2017). In 2–16% of cases, spina bifida is associated with chromosomal abnormalities (frequently trisomy 18) (Sepulveda et al., 2004) and pathogenic Copy Number Variants (CNVs) at Chromosomal Microarray Analysis (CMA) are found in 3.7% of cases (Santirocco et al., 2021). In a small proportion of cases, the open NTD can be a feature of monogenic syndromes, including Jarcho–Levin, cerebrocostomandibular, Neu–Laxova, DiGeorge, SDAM (sacral defect with anterior meningocele), Czeizel–Losonci, Weissenbacher–Zweymüller, pentalogy of Cantrell, and OEIS (omphalocele–exstrophy–imperforate anus–spinal defects) complex (Chen, 2008). Many of these disorders can be confirmed by CMA and exome sequencing (ES). An increase in alpha-fetoprotein concentrations in maternal serum and amniotic fluid is a typical feature of open NTD and needs fetal neurosonography. Prenatal diagnosis of open NTD should aim at detecting the associated CNS and systemic anomalies. In *utero* surgical repair was initially reported to improve postnatal motor and mental functional scores. However, in a recent systematic review and meta-analysis on the neurodevelopmental outcome of children with open spina bifida, the risk of neurodevelopmental impairment was similar in those who were repaired prenatally and postnatally, although the cases operated in *utero* had an increased risk of prematurity (Inversetti et al., 2019). In the United States, approximately 90% of babies born with open spina bifida survive the first year of life, and 75–80% survive to early adulthood (Trudell & Odibo, 2014). The outcome of closed spina bifida is significantly better than that of open spina bifida. The majority of postnatally diagnosed closed spina bifida cases are incidentally detected as having occult spina bifida without a subcutaneous mass (Leibovitz et al., 2022).

1.1.3.2 Anomalies of Ventral induction: Holoprosencephaly

Holoprosencephaly (HPE) is caused by anomaly in ventral induction, resulting by abnormal cleavage of prosencephalon into two telencephalic vesicles between the 6th and 9th postmestrual weeks, forming the cerebral hemispheres. The most accepted classification of HPE comprises alobar, semilobar, and lobar HPE forms, based on the degree of failure of prosencephalic cleavage (Demyer et al., 1964).
In alobar HPE (no cleavage), a single monoventricular forebrain is formed. The interhemispheric fissure and the falx cerebri, the corpus callosum (CC), and the cavum septi pellucidi (CSP) are absent. Alobar HPE is typically associated with fusion of the basal ganglia, thalami, and choroid plexuses. Differentiation of hydranencephaly and severe hydrocephalus from alobar HPE is based on normal thalamic cleavage and visualization of the interhemispheric fissure and falx cerebri (Winter et al., 2015). Alobar HPE is associated with the most severe facial malformations, affecting the development of the eyes, nose, upper lip, and palate. The eyes and optic nerves, and olfactory bulbs

and tracts may be separated, fused, or absent. The ophthalmologic features include cyclopia (single central eye), synophthalmia (two fused eyes in the midline), hypotelorism, and colobomas. Nasal defects can manifest as a lack of a nose with a proboscis (ethmocephaly) or as a small nose with a single nostril (cebocephaly). The oral anomalies of alobar HPE may manifest with a midline cleft lip and/or palate (Winter et al., 2015).

In semilobar HPE (partial hemispheric cleavage), the frontal lobes are fused, but the parieto-occipital regions are divided by the interhemispheric fissure and falx cerebri (Demyer et al., 1964). The CSP is absent, the posterior part of the CC may form. The thalami are usually fused, and facial malformations are usually milder compared to alobar HPE (Winter et al., 2015).

In lobar HPE (almost complete hemispheric cleavage), only the inferior frontal lobes are fused, and the majority of the interhemispheric fissure and falx cerebri are well-formed along the midline and the thalami are usually separated. These characteristics make diagnosis of lobar HPE more difficult than alobar and semilobar HPE, reliably diagnosed by the end of the first trimester of pregnancy. Milder forms may be associated with septo-optic dysplasia and a nonseparation of the preoptic area, respectively. The mildest form is characterized only by facial features (hypotelorism and single maxillary central incisor) and normal brain development (Leibovitz et al., 2022).

Other extracranial abnormalities are present in 46% and are dependent on the etiology. They include urogenital anomalies (24%), postaxial polydactyly (8%), vertebral defects (5%), limb reduction defects (4%), and transposition of great arteries (4%) (Orioli & Castilla, 2010).

HPE is the most common developmental disorder of the forebrain, occurring in about 1/250 of conceptuses but is much rarer (0.4-1.2/10 000) in live births as the majority of fetuses with HPE do not survive the pregnancy (Orioli & Castilla, 2010; Van den Veyver, 2019). It can be caused by genetic and environmental factors (Chaudhari & Ho, 2022). Aneuploidy account for 25-55% of fetuses diagnosed with HPE of which 75% are trisomy 13 and others include triploidy (20%) and trisomy 18 (1%-2%) (Winter et al., 2015).

The prevalence of pathogenic CNVs in syndromic HPE varies in literature between 10% and 22% (Levey et al., 2010), whereas single Nucleotide variants (SNVs) accounts for 18-25% of cases; of which 10% are related to abnormalities of cholesterol biosynthesis. The most common among these is SLOS (Smith-Lemli-Opitz Syndrome), an autosomal recessive disease of variable severity, caused by biallelic pathogenic variants in *DHCR7,* the gene encoding 7-dehydrocholesterol reductase. Features of SLOS include fetal growth restriction, microcephaly, occasionally HPE, cleft palate, cardiac defects, hypospadias or ambiguous genitalia in males, two to three syndactyly of the toes, postaxial polydactyly, distinctive facial features, and the potential for severe neurodevelopmental disability. Recently, expanded carrier screening has revealed a higher carrier frequency for *DHCR7*

variants than previously ascertained, suggesting that there may be high early prenatal lethality of affected fetuses (Lazarin et al., 2017). Other monogenic syndromic conditions include CHARGE (related to heterozygous pathogenic variants in *CHD7*), Pallister Hall (related to heterozygous pathogenic variants in *GLI3*), Rubinstein-Taybi (related to heterozygous pathogenic variants in *CREBBP* or *EP300*), Velocardiofacial (related to heterozygous pathogenic variants in *TBX1*), Meckel Syndrome and Aicardi syndromes (Kauvar & Muenke, 2010; Dubourg et al., 2018).

Variants in *SHH, ZIC2, SIX3, TGIF, GLI2, FGF8,* and *FGFR1* genes can be detected in about 20% of non-syndromic isolated HPE cases with a normal karyotype (Roessler et al., 2018). *SHH* mutations are the most common cause of this group which can be detected in 40% of familial and <5% of sporadic cases. *ZIC2, SIX3,* and *TGIF1* are present in 5%, 1.3%, and 1.3% of familial cases, respectively, and more rarely *GLI2, PTCH1, DISP1, FGF8, FOXH1, NODAL, TDGF1, GAS1, DLL1* (Roessler et al., 2018). They manifest with variable penetrance and lead to classic HPE forms in only 50% of the patients. Parental hypotelorism or a single maxillary central incisor can be the only indication of the presence of a familial *SHH* pathogenic variant in apparently unaffected family members (Kauvar & Muenke, 2010, Kruszska & Muenke, 2018). Thus, examining parents is helpful for recurrence risk counseling.

Nongenetic etiologies of HPE include uncontrolled pregestational maternal diabetes mellitus (carries 1% of risk), maternal alcohol intake, and embryo exposure to retinoic acid (Van den Veyver, 2019; Leibovitz et al., 2022).

HPE is associated with high postnatal mortality. Only a small proportion of children with HPE survive into adulthood. Survival reaches better rates in non-syndromic, euploid HPE patients. The severity of brain and facial defects is inversely correlated with survival. Almost all children with classic forms of HPE have abnormal neurodevelopment, which correlates with the severity of the CNS findings. Typically, alobar HPE results in profound global developmental impairment. Patients with semilobar, lobar HPE, can develop variable degrees of motor and speech skills (Levey et al., 2010)

1.1.3.3 Midline Defects: Corpus Callosum Anomalies and Cavum Septum Pellucidum

CC anomalies have a prevalence estimated between 0.3% and 0.7% in postnatal Magnetic Resonance Imaging (MRI) (Edwards et al., 2014), and in n up to 0.03% to 0.07% of second trimester ultrasounds (US) (Van den Veyver, 2019), representing the most frequent cerebral malformations observed in humans. The basic structural parts of the CC (rostrum, genu, body, and splenium) are formed by mid-gestation, reaching full maturity only in adolescence, with typical thinning of the isthmic segment between the body and the splenium (Leibovitz et al., 2022; Scelsa, 2022).

Abnormal callosal development may be caused by defects in neuronal and glial proliferation, midline patterning, neuronal migration and specification, axonal growth and guidance, and post-guidance development (Edwards et al., 2014). After crossing the midline, the callosal axons arrive mainly to homotopic contralateral regions, and fetal CC elongates antero-posteriorly in a bidirectional pattern, with the rostrum and the splenium being the last to form (Birnbaum et al., 2020).

CC anomalies constitute a broad spectrum of commissural defects which include complete agenesis of the corpus callosum (cACC, in approximately 1/4000 live births), partial agenesis (pACC), hypoplasia (complete and thin corpus callosum) and hyperplasia (complete and thick corpus callosum) and dysgenesis (abnormally shaped CC) (Leombroni et al., 2018).

A variety of genetic, metabolic, disruptive, and toxic causes (*i.e.* alcohol intake or congenital infections) can result in abnormal CC development; however, the etiology of CC abnormalities are still unknown in many cases (Diogo et al., 2021). There are more than a thousand different syndromes and metabolic disorders associated with CC anomalies, most of them leading to moderate to severe neurodevelopmental disability (des Portes et al., 2018).

High-resolution fetal neurosonography performed at the second half of gestation enables accurate imaging of the fetal CC. Callosal anatomy can be optimally demonstrated in the mid-sagittal plane, and Color Doppler imaging of the pericallosal artery above the CC can improve callosal detection (Birnbaum et al., 2020).

In 45-50% of fetuses with ACC, additional CNS abnormalities may be detected. The most common associated anomalies are malformations of cortical development (polymicrogyria, lissencephaly, pachygyria, schizencephaly) and posterior fossa defects. Destructive parenchymal brain lesions have been reported in 10% of ACC fetuses (Tang et al., 2009). Associated abnormalities of the Sylvian fissure and the hippocampus have been reported to aggravate the neurodevelopmental outcome (Diogo et al., 2021). Extra-CNS abnormalities are present in 65% of cases (Mangione et al., 2011). CMA and ES enable diagnosis of the underlying genetic etiology of CC abnormalities in approximately 20% of prenatal cases (Heide et al., 2020).

In apparently isolated fetal ACC, the risk of ACC being not truly isolated is relatively high, with additional anomalies detected only at postnatal imaging and/or clinical examination reported to occur in 5.5% (95% CI 2.4-9.7) and 15.0% (95% CI 6.7-24.6), respectively (Leombroni et al., 2018). According to a meta-analysis performed by D'Antonio et al., a normal neurodevelopmental outcome is expected in 76.0% (95% CI 64.3–86.1%) and 71.4% (95% CI 53.1–86.7%) of children with prenatally diagnoses isolated cACC and pACC, respectively (D'Antonio et al., 2016).

A fetal brain MRI anatomical score based on evaluation of gyration, opercularization, temporal lobe symmetry, parenchymal lamination, hippocampal position, basal ganglia, and ventricular size, has

recently been developed to improve prognostication of postnatal neurodevelopmental outcome (Diogo et al., 2021).

The CSP is visible as early as 15 weeks and should be seen between 18 and 37 weeks, after which it begins to narrow, if the CSP is not seen by 18 to 20 weeks' gestation, further evaluation, including MRI, is indicated to search for other brain abnormalities (Sundarakumar et al., 2015).

Absent CSP is rare and usually represents an early sign of other midline forebrain anomalies, such as HPE and ACC, but it can be also a normal variant with good prognosis (Pilliod et al., 2018).

When frontal horns are small, partial or complete ACC with or without other migrational abnormalities can be present. When frontal horns are normal and there is a CC, it could indicate septo-optic dysplasia, better referred to as hypoplastic optic nerve syndrome (Pilliod et al., 2018).

This spectrum of abnormalities includes hypoplastic optic nerve, which may require postnatal ophthalmologic exam for confirmation, abnormalities of the hypothalamic-pituitary axis, and in some, ACC and schizencephaly (Sundarakumar et al., 2015). Genetic causes are found in only 1% of cases, including variants in *HESX1, SOX1, SOX2* (with severe eye defects and other anomalies), *SOX3, OTOX2*, and *FGF8* (Van den Veyver, 2019).

The significance of an enlarged (>10 mm) CSP is controversial, but it can be a sign of a CSP cyst and associated with other findings, including chromosomal abnormalities (Ho et al., 2017).

1.1.3.4 Malformations of Cortical Development

Cortical development is governed by three overlapping processes: neuronal and glial proliferation, neuronal migration, and post-migration neuronal development and organization (Fernàndez et al., 2016). Depending on the underlying mechanism, a vast spectrum of morphological abnormalities can result with focal, segmental, or diffuse patterns.

Malformations of cortical development (MCD) are a heterogeneous group of malformations related to disorders in one or more of these processes and MCD are classified according to the earliest regulating process affecting cortical development (Desikan & Barkovich, 2016).

Cortical malformations, especially focal defects, such as heterotopia, polymicrogyria, and focal cortical dysplasias are often not visible until later in gestation, often after the routine 20-week morphological US, and their diagnosis requires neurosonography and fetal MRI expertise.

Typical imaging features are abnormal (pre-mature, delayed, absent, or wide) sulcation, thin or thickened irregular hemispheres with abnormally developed and wide gyri, cortical clefts, or heterotopia, which present as nodular protrusions into the lateral ventricles or as intraparenchymal nodules (Van denVeyver, 2019).

The neurosonographic signs suggestive of fetal MCD include abnormalities of the Sylvian fissure, delayed cortical sulcation, prematurely appearing or aberrant sulci, ventricular wall irregularity, abnormal parenchymal thickness, abnormal shape and orientation of the sulci and gyri, hemispheric asymmetry, simplified gyral pattern, cortical cleft, and parenchymal echogenicities (Leibovitz et al., 2022). Pregnant women with family history of MCD, cases of intrauterine infection, and fetuses with ventriculomegaly, abnormal head circumference (HC), callosal disorders, cerebellar, vermian, or brainstem abnormalities should be evaluated for MCD.

When fetal MCD is suspected, a dedicated neurosonography and a detailed systemic anatomical scan should be performed. Serological tests for intrauterine infection, fetal head MRI, and CMA as well as ES are recommended. Parents should be referred for multidisciplinary counseling by specialists in pediatric neurology, genetics, neurosonography, and MRI (Lerman-Sagie et al., 2021).

1.1.3.4.1 Disorders of abnormal neurogenesis (proliferation)

Abnormal neurogenesis refers to decreased or increased proliferation or apoptosis of neurons and glia. Genetic anomalies or insults that affect cell proliferation will lead to reduced or excessive growth, causing microcephaly or megalencephaly, respectively (Leibovitz et al., 2022).

Primary microcephaly, present at birth, also known as true microcephaly or microcephaly *vera*, results from decreased proliferation or increased apoptosis of neural and glial cells. It is defined by the clinical finding of a HC more than 3 standard deviations (SD) below the age- and sex-related population mean (Desikan & Barkovich, 2016) and it is measured through US around the outer borders of the fetal skull in the transthalamic axial head plane (Leibovitz & Lerman-Sagie, 2018).

Secondary microcephaly may develop in the post-neurogenesis period due to disruptive events such as ischemia, infection, and trauma, inborn errors of metabolism, teratogens, and genetic disorders. Postnatal microcephaly is defined as a HC more than 2 SD below the mean for age and gender and it is measured with a centimeter tape, including the scalp and hair. The difference in methodology between prenatal and postnatal HC evaluation reduces the accuracy of fetal microcephaly diagnosis. The positive prediction of microcephaly at birth based on sonographic HC evaluation in utero ranges between 57% and 67% (Leiboviz et al., 2016).

Genetic causes of microcephaly include chromosomal abnormalities and pathogenic CNVs (present in 5.4%) (Shaffer et al., 2012). Many genes have been associated with isolated primary microcephalies, many of which encode proteins with essential roles in chromosomal segregation, mitotic neural and glia cell division, centrosome, and kinetochore function. Others have a role in DNA damage repair, DNA replication, and cilia function (Duerinckx &Abramowicz, 2018).

Genes responsible for primary microcephaly include *MCPH1, ASPM, CDK5RAP2, CENPJ, STIL, WDR62, CEP152,* and *CEP63*, and have autosomal recessive inheritance (Jayaraman et al., 2018).

A distinct group of syndromic disorders with microcephaly include autosomal recessive microcephalic primordial dwarfisms, which include Seckel syndrome caused by pathogenic variants in *ATR* and *ATRIP* and centriole biogenesis genes.

Another distinct condition is Meier-Gorlin syndrome caused by variants in *ORC1, ORC4, ORC6,* and other genes that play a role in origin of DNA replication.

A third class of syndromes are the microcephalic osteodysplastic primordial dwarfisms (MOPDs) (Duerinckx &Abramowicz, 2018; Jayaraman et al., 2018). MOPD1 is caused by pathogenic variants in the noncoding RNA gene *RNU4ATAC*, which codes for small nuclear RNA U4atac, a component of the minor spliceosome. If ES is performed for genetic work-up and this noncoding gene is not included in the exome capture library, these variants can be missed (Wang et al., 2018).

MOPD2 is caused by pathogenic variants in *PCNT*, which encodes pericentrin, an essential component of the pericentriolar material in the centrosome and essential for normal mitotic spindle formation. The genetic etiology for MOPD3 is still unknown (Van den Veyver, 2019).

The developmental prognosis of congenital microcephaly is influenced by the presence of other associated intracranial and extra-cranial abnormalities and negatively correlates with the size of the brain. In −2SD HC, 10% have intellectual disability and in −3SD HC, 50% have severe intellectual disability (Mighell et al., 2009). Data reporting the correlation between the severity of prenatal microcephaly and the prevalence of neurodevelopmental disability after birth are very limited, making prognosis for fetal microcephaly difficult (Leibovitz & Lerman-Sagie, 2018).

Megalencephaly refers to brain overgrowth due to excessive proliferation or decreased apoptosis of neural and glial cells. Macrocephaly is defined as an HC that is at least 2 SD above the mean in both pre- and post-natal evaluations (Pavone et al., 2017).

Megalencephaly can be primary or secondary (Mirzaa & Poduri, 2014). It is rare to diagnose syndromic megalencephaly prenatally, in most cases, megalencephaly manifests only postnatally (Leibovitz et al., 2022).

Primary megalencephaly results from a cellular defect that causes a more general overgrowth syndrome or can result in overgrowth of the only brain, which can be generalized, confined to a single hemisphere (hemimegalencephaly), or focal (focal cortical dysplasia).

Activating dominant or somatic mutations in genes that control two main pathways with important roles in cell growth regulation, RAS-MAPK (RASopathies) and PI3K-AKT-mTOR, have been implicated in these conditions (Mirzaa & Poduri, 2014).

Syndromic megalencephaly may be associated with overgrowth syndromes (Sotos, Weaver, Simpson–Golabi–Behmel), megalencephaly-capillary malformation, megalencephaly–polymicrogyria–polydactyly–hydrocephalus, and hemimegalencephaly.
Secondary megalencephaly can be a sign of metabolic disorders that affect the brain, such as Canavan disease, glutaric aciduria type I, and lysosomal storage disorders. These are usually gradually progressive postnatally and often not detected by prenatal imaging (Mirzaa & Poduri, 2014). Prenatally diagnosed macrocephaly can be familial, isolated, and benign.
Several disorders other than megalencephaly can cause macrocephaly, including hydrocephalus and enlargement of the subarachnoid spaces, brain tumor, and massive subdural hemorrhage. Macrocephaly can also accompany skeletal dysplasias (achondroplasia, thanatophoric dysplasia, Apert syndrome) (Leibovitz et al., 2022).

1.1.3.4.2 Disorders of abnormal neuronal migration

MCD secondary to abnormal neuronal migration primarily refer to lissencephaly, cobblestone malformation, and gray matter heterotopia. Early prenatal detection is challenging because neuronal migration and cortical folding occurs in the late second and third trimester of fetal development and because of the limitations of US, therefore Fetal MRI is needed (van den Veyver, 2019).
The term lissencephaly is derived from the Greek words *"lissos"* (smooth) and *"enkephalos"* (brain) and it is divided into classic or type I lissencephaly and cobblestone or type II lissencephaly.
Type-1-lissencephaly is caused by neuronal under-migration. Cerebral cortex is characterized by a smooth thick, disorganized four-layer cortex and absence of deep sulci and gyri, with absence of other major brain anomalies. It can be divided into six grades from mild (grade 6) to complete agyria (grade 1) (Romero et al., 2018; Severino et al., 2020). The term pachygyria is also used to indicate a few broad gyri and shallow sulci.
Pathogenic variants of multiple genes are associated to lissencephaly, most of them being related to tubulin and microtubule-associated proteins. The MRI classification of lissencephaly is based on the grade of cortical smoothness, the gradient of the gyration, cortical thickness, and associated brain abnormalities. This classification allows genotype-phenotype correlations (Kato & Dobyns, 2003). Genetic anomalies causing lissencephaly can be sporadic, autosomal dominant (usually *de novo*), autosomal recessive, or X-linked. There are three main genes responsible for classic lissencephaly: The first is *LIS1 (PAFAH1B1)* in 17p13.3 locus. *LIS1* loss of function variants, cause Miller-Dieker syndrome. This syndrome is often caused by a microdeletion, which is de novo in 80% of cases and inherited from a parent with a balanced translocation in 20% of cases; however also SNVs in *LIS1* have been reported.

On late second and third trimester neurosonography, lissencephaly manifests with a bilaterally reduced or absent sulcation. The fetal brain has a typical figure "8" shape in the coronal transthalamic plane. *LIS1* lissencephaly is characterized by a lack of occipital gyration and a relatively spared frontal gyration (a posterior to anterior gradient).

The second commonest cause of classic lissencephaly is related in males to pathogenic variants in *DCX* in Xq22.3 locus, of whom 25% inherited from their mother and the remainder caused by *de novo* variants (Romero et al., 2018). This type of lissencephaly present with an anterior to posterior gradient. In contrast, lissencephaly associated with microcephaly should raise suspicion for *TUBA1A* variants or other genes implicated in tubulinopathies (*TUBB2B, TUBG1, etc)*. In lissencephaly with an anterior to posterior gradient and severe cerebellar hypoplasia, genetic variants in *RELN*, *CDK5*, or *VLDLR* should be suspected, whereas a posterior to anterior gradient with ACC can be related to *ARX* or *TUBA1A* variants (Kato & Dobyns, 2003).

Cobblestone cortical malformation, formerly classified as type-2 lissencephaly, is caused by neuronal and glial over-migration. This phenotype is associated with a very disorganized cortex with multiple coarse gyri and areas with no gyri (*i.e.* a more nodular cerebral cortical surface) together with other CNS abnormalities, including abnormal pons and kinked brain stem, cerebellar abnormalities, and callosal abnormalities (Van den Veyer, 2019). This malformation is related to abnormal O-glycosylation of α-dystroglycan. Pathogenic variants in *POMT1, POMT2, POMGnT1, FKTN, FKRP, LARGE, B3GALNT2, B3GNT1, GALNT2, GTDC2, ISPD,* and *TMEM5* lead to a spectrum of autosomal recessive disorders (α-dystroglycanopathies) that may manifest with cerebral, ocular, and muscular abnormalities (Walker–Warburg syndrome, muscle–eye–brain syndrome, and Fukuyama muscular dystrophy) (Desikan & Barkovich, 2016).

The most severe type of cobblestone cortical malformation is Walker–Warburg syndrome. On neuroimaging, the cerebral parenchyma is thin, and the cortex is mildly thickened, depicting radial striation and a serrated interface between the gray and white matter. Severe ventriculomegaly, cerebellar and vermian hypoplasia, and a "Z"-shaped hypoplastic brainstem are consistent features of Walker–Warburg syndrome (Severino et al., 2020). Cephalocele, retinal dysplasia, microphtalmia, and congenital muscular dystrophy can be associated with this syndrome.

Disorders of incomplete migration can also cause heterotopias. Gray matter heterotopias refer to clusters of normal neuronal cells abnormally located in the periventricular zones, the white matter, or the outer cortical margins, all related to impaired migration (Leibovitz et al, 2022).

The most common forms of heterotopias are periventricular heterotopias caused by neurons which do not migrate out of the periventricular zone and subcortical band heterotopia caused by incomplete

neurons' migration along the trajectory to the cerebral cortex. This defect creates a bandlike layer of neurons within the white matter, referred to as *"double cortex"* (Leibovitz et al., 2022).

Subcortical band heterotopia is caused by heterozygous variants in females in the X-linked *DCX* gene, encoding doublecortin. Affected girls and women have focal or generalized seizures and normal intelligence or mild learning disability, or intellectual disability of variable severity, depending on the type of variant, X-inactivation pattern, and mosaicism (Romero et al., 2018). When women transmit the *DCX* pathogenic variant to males, it causes classic lissencephaly (Van den Veyver, 2019).

A common form of periventricular heterotopias is bilateral periventricular nodular heterotopia caused by variants in *FLNA* gene in 49% of all cases. In this X-linked dominant condition, females are variably affected because of mosaicism or X-inactivation patterns.

Loss of function *FLNA* variants are prenatally lethal in males, but there are surviving males carrying hypomorph and mosaic mutations. Bilateral periventricular nodular heterotopia is typically detected by MRI that reveals bilateral nearly contiguous periventricular heterotopias at the lateral ventricular walls with an otherwise normal appearing cortex. The CC can be thin, and there can be posterior fossa and cerebellar abnormalities. Periventricular heterotopia presents with a specific *"string of pearls"* sign and an enlarged cisterna magna. It clinically manifests primarily as a seizure disorder in affected girls and women, who can have from normal intelligence to borderline intellectual disability and may have higher risk for cardiovascular disease.

A rarer recessive form of bilateral periventricular nodular heterotopia is caused by biallelic variants in *ARFGEF2*, usually associated with microcephaly and a poor prognosis (Lerman-Sagie et al., 2021).

Tuberous Sclerosis should be considered if periventricular nodules are identified on prenatal ultrasound. Prenatal intracranial features include cortical tubers in 70% and subependymal nodules in 90%, but the most prominent prenatal findings are single or multiple cardiac rhabdomyomas.

In contrast to CNS findings, these often regress after the neonatal period, but their discovery should prompt detailed evaluation of the brain. Tuberous Sclerosis is highly clinically variable: 80% of affected individuals have seizures and 50% have neurodevelopmental and behavioral impairment. Among subjects satisfying clinical criteria for this condition, 85% have autosomal dominant heterozygous mutations in either *TSC1* (31%) or *TSC2* (69%), which is the more severe form.

Two-thirds have *de novo* variants, and one-third are familial. Germline (in 5%) and somatic mosaicism (in 1%) have both been reported in Tuberous Sclerosis, and this aspect should be considered in recurrence risk counseling (Van den Veyver, 2019).

1.1.3.4.3 Postmigrational defects (Disorders of Cortical Disorganization)

Postmigrational defects that cause cortical disorganization result in polymicrogyria and focal cortical dysplasias. These anomalies develop in the late second and third trimesters of pregnancy, affecting the formation of the six-layered cortex, outgrowth of axons and dendrites from cortical neurons, and synaptogenesis (Leibovitz et al., 2022).

Gyration abnormalities can be isolated or associated with other intracranial and extracranial abnormalities. They include polymicrogyria, agyria, and pachygyria and constitute 20% of all malformations of cortical development. They can be difficult to see on prenatal ultrasound and are best evaluated by MRI, performed in the late second or early third trimester (after 20-22 weeks). Polymicrogyria can be focal, diffuse, unilateral, or bilateral and it is characterized by an excessive number of small gyri separated by shallow sulci, giving the cortex an irregular, bumpy surface with apparent thickening, an irregular gray–white matter interface, and a prominent subarachnoid space overlying the affected cortex (Van den Veyver, 2019).

On prenatal neuroimaging, it appears as a focal or diffuse cortical irregularity or multiple small infoldings of the affected cortex and most frequently affects the Sylvian fissure depicting an abnormal opercular contour (Leibovitz et al., 2022).

Recent studies have suggested that an abnormal shape and size of the sylvian fissure in early pregnancy can be an early sign of migration abnormalities and indication for further imaging by US and MRI at a later gestational age (Pooh et al, 2019).

Polymicrogyria has a multifactorial pathogenesis including chromosomal abnormalities, monogenic cause, intrauterine infection, exposure to teratogens, and damage due to ischemia or vascular disruption. The most common CNV associated with polymicrogyria are 22q11.2 deletion and 1p36 deletion. SNVs in more than 40 genes have been related to polymicrogyria, caused by a variety of autosomal dominant (usually *de novo*), autosomal recessive, and X-linked single-gene variants.

These include metabolic and lysosomal disorders, such as Zellweger syndrome, neonatal adrenoleukodystrophy, Pelizaeus-Merzbacher Syndrome, glutaric aciduria type 2, maple syrup urine disease and can be also related to genes of the PI3K-AKT-MTOR pathway, tubulinopathies, and α-dystroglycanopathies (Lerman-Sagie et al., 2019).

For some conditions, such as Aicardi syndrome, the genetic cause is still unknown. This syndrome tipically affects females only and is suspected when there are polymicrogyria, heterotopias, and cysts, but it can only be confirmed after birth by demonstration of the typical chorioretinal lacunae on fundoscopic eye examination.

In patients with polymicrogyria, the prevalence of epilepsy and global developmental delay is high, reaching 78% and 70%, correspondingly (Leventer et al., 2010).

Schizencephaly is a subtype of polymicrogyria presenting as a cleft in the cerebral cortex that connects the subarachnoid space to the ventricles. It can have an open (type II) or closed lip (type I) and be unilateral or bilateral and is typically lined by unlayered polymicrogyria (Romero et al., 2018; Lerman-Sagie et al., 2021). In type II, the missing parenchyma is replaced by CSF and can be clearly demonstrated on a prenatal brain scan. In type I, the lips of the cleft face each other closely, making detection difficult (Severino et al., 2020). The neurologic manifestations of schizencephaly depend on the type and location of the lesion. It is believed to be a result of an early disruptive injury to the developing cerebral hemispheres (Leibovitz et al., 2022).

1.1.3.5 Cerebellar and Posterior Fossa Abnormalities

The posterior fossa (PF) contains the brainstem, cerebellum, and surrounding CSF spaces. Cerebellar abnormalities affect 1:5000 live births and are often associated with other CNS and non-CNS anomalies (Van den Veyver, 2019). The tentorium separates the PF structures from the occipital lobes. The upper part of the brainstem (the midbrain) is derived from the mesencephalon, while the pons, cerebellum, and medulla oblongata develop from the rhombencephalon (Leibovitz et al., 2022). Development of the cerebellar folia and vermian lobulation can be reliably assessed on prenatal neuroimaging only by late second trimester. Therefore, many PF malformations cannot be correctly diagnosed earlier (Garel et al., 2011). Hereinafter common examples of PF malformations will be treated specifically.

1.1.3.5.1 Dandy-Walker malformation and Vermian anomalies

Although terms like Dandy-Walker variant, Dandy-Walker spectrum, or Dandy-Walker complex were previously used, it is now suggested to abandon these designations for the more descriptive terminology of Dandy-Walker malformation (DWM) (Van den Veyver, 2019).

The diagnosis of DWM is based on three imaging features: (1) an enlarged posterior fossa with upward displacement of the tentorium cerebelli; (2) cystic dilatation of the fourth ventricle that fills nearly the entire retrocerebellar space; and (3) partial or complete vermian agenesis and substantial upward rotation of the vermis (Guibaud, 2004).

DWM occurs in 1:25,000–1:35,000 births. Associated chromosomal abnormalities (mainly trisomy 18 and 13) can be detected in 16-35% of cases, as well as a variety of monogenic disorders (D'Antonio et al., 2016). Up to 86% of fetuses with DWM have other abnormalities of which 30–50% of cases, DWM accompanies other CNS anomalies, including ACC, occipital encephalocele, polymicrogyria, gray matter heterotopias, and holoprosencephaly.

Associated systemic anomalies include facial clefts, congenital heart defects, urinary malformations, and polydactyly and DWM occurs in numerous syndromes (*i.e.* Walker–Warburg, Meckel–Gruber, Aicardi, Neu–Laxova, *etc*). Deletions or loss of function variants in three transcription factors (*FOXC1, ZIC1* and *ZIC4)* are the best described, but account for only 5% of cases (Guibaud, 2004). The differential diagnosis of DWM includes other conditions leading to cystic dilatation of the posterior fossa: Blake's pouch cyst, mega cisterna magna, PF arachnoid cyst, and destruction of cerebellar tissue after a clastic insult (Severino & Huisman, 2019).

Development can be normal in up to a third of prenatally detected DWM; in particular, when the vermis is normally lobulated, 82% to 90% have normal intelligence.

However, with associated CNS or extra-CNS malformations or absent or abnormal vermis lobulation, 50% will have neurological abnormalities, 50% will have hypotonia, 42% will have cerebellar dysfunction, and 5% will show hemiparesis (Van den Veyver, 2019).

Vermian agenesis, hypoplasia, and dysgenesis are other common PF anomalies. Vermian agenesis refers to the complete or partial absence of vermian tissue. In partial vermian agenesis, the missing part is typically the inferior one, due to cranio-caudal development of the vermis (Guibaud, 2004). Vermian hypoplasia refers to a small but completely formed vermis without missing lobules. Differentiation between vermian hypoplasia and vermian agenesis in utero is difficult due to imaging limitations. On postnatal MRI, vermian hypoplasia is frequently described with abnormal vermian lobulation and a dysmorphic fastigial shape (Severino & Huisman, 2019) but the term vermian dysgenesis is more appropriate for description of such findings (Leibovitz et al., 2022).

Despite the difference in definitions, the terms vermian agenesis, hypoplasia and dysgenesis are interchangeably used in the literature causing inaccuracy of prognostication (D'Antonio et al., 2016 part 1 and part 2).

Due to late vermian maturation, the diagnosis of vermian abnormalities can remain unclear prior to 24 weeks. Inferior vermian agenesis and dysgenesis may be associated with intellectual disability and autistic spectrum disorder.

Information regarding abnormal neurodevelopmental outcomes in children with a prenatal diagnosis of vermian anomalies is limited. According to a recent meta-analysis, the mean rate of abnormal neurodevelopmental outcomes after prenatal diagnosis of isolated vermian hypoplasia was 30.7%; however, it was not statistically significant due to the very small number of the studied cases (D'Antonio et al., 2016).

Noteworthy, inferior vermian anomalies can be the only fetal malformation in syndromic conditions, and accurate fetal screening and a postnatal follow-up is recommended (Scelsa, 2022).

1.1.3.5.2 Blake's Pouch Cyst

During its development, PF undergoes numerous changes, including the appearance and disappearance of several structures. One of the most significant ones is Blake's pouch which disappears at approximately 18 gestational weeks (GW) with the closure of the fourth ventricle (Scelsa, 2022). Persistence of Blake's Pouch Cyst is thought to be due to delayed fenestration of the posterior membranous area of the developing fourth ventricle (Leibovitz et al., 2022).

The differential diagnosis of Blake's pouch with other conditions, such as an arachnoid cyst, mega cisterna magna, cerebellar hypoplasia/vermian hypoplasia, and DWM can be challenging for the prognostic implications (Kau et al., 2020).

The prognosis of Blake's pouch tends to be favorable in most cases, however, the same does not always apply to the other conditions. Measurement of the brainstem–vermis angle has been proposed for differentiating between Blake's pouch cyst, inferior vermis agenesis, and DWM.

The risk of abnormal neurodevelopment following prenatal diagnosis of isolated Blake's pouch cyst is low (4.7%) and the risk of chromosomal anomalies is 5.2%. An association with additional CNS anomalies after birth has not been found (D'Antonio et al., 2016). However, in the case of additional structural (CNS and extra-CNS) or genetic abnormalities, the incidence of neurodevelopmental disabilities has been described in 20% of the cases, and genetic testing should always be offered (Society of Maternal-Fetal Medicine, 2020).

1.1.3.5.3 Joubert Syndrome (JS) and JS-Related Disorders

This group of rare disorders is caused by abnormal function of the primary nonmotile cilia (ciliopathies). The cilia are implicated in neuronal cell proliferation and axonal guidance. More than 30 ciliopathy genes are actually known, most of which have autosomal recessive inheritance, and a few X-linked transmission (Severino & Huisman, 2019). ES yields a molecular diagnosis in 62% to 94% of cases (D'Antonio et al., 2016).

In this group of overlapping conditions, the cerebellar/posterior fossa abnormality is characterized by an abnormally deep interpeduncular fossa, more horizontally oriented, enlarged superior cerebellar peduncles, and hypoplastic cerebellar vermis. This combination of findings results in the pathognomonic molar tooth sign, which is easily recognizable with fetal MRI, but can also be observed by high-resolution prenatal US (Lerman-Sagie et al., 2018).

Brainstem and supratentorial abnormalities (polymicrogyria, ACC, gray matter heterotopia, and occipital cephalocele) may accompany JS in about 30% of cases (Severino & Huisman, 2019).

Children with JS present with hypotonia, developmental delay, ataxia, eye movement abnormalities, and abnormal breathing.

JS-related disorders are characterized by additional abnormalities such as coloboma, liver fibrosis, tongue tumors, polydactyly, nephronophthisis, retinal dysplasia, and cystic dysplastic kidneys (Leibovitz et al., 2022). There is significant overlap with other conditions such as Meckel-Gruber syndrome, Hydrolethalus syndrome, nephronophthisis syndrome, acrocallosal syndrome, Bardet-Biedl syndrome, and orofacial digital syndrome (Lerman-Sagie et al., 2018).

1.1.3.5.4 Mega cisterna magna

Mega cisterna magna or a transverse measurement of the posterior fossa >10 mm in the anteroposterior dimension with normal appearing cerebellar vermis and hemispheres, is the most common prenatally diagnosed PF abnormality, comprising 40% of all findings (Massoud & Guibaud, 2018). The neurodevelopmental outcome is normal in 86% to 100% of isolated cases (D'Antonio et al., 2016; Massoud & Guibaud, 2018). Additional CNS abnormalities are detected on 12.6% of cases (most commonly ventriculomegaly), and extra-CNS anomalies are found in 16.6% (D'Antonio et al., 2016). Small series suggest that when mega cisterna magna is associated with other CNS and non-CNS defects, up to two-thirds develop normally and one-third have cognitive, language, and motor delay and have an increased risk for chromosomal or other genetic abnormalities (Van den Veyver, 2019).

1.1.3.5.5 Rhombencephalosynapsis

Rhombencephalosynapsis is defined as median fusion of the cerebellar hemispheres, dentate nuclei, and cerebellar peduncles associated with a complete or partial absence of the vermis. The genes responsible for this malformation are unknown, but it is usually a sporadic malformation, or it may be part of Gomez–Lopez–Hernandez syndrome, VACTERL (vertebral defects, anal atresia, cardiac defects, tracheo-esophageal fistula, renal anomalies, and limb abnormalities), or HPE (Krajden Haratz & Oliveira Szejnfeld). Rhombencephalosynapsis may be complicated by hydrocephalus. In fetuses with rhombencephalosynapsis, the cerebellum is small and round-shaped, the posterior cerebellar notch is absent, and the folia run continuously on the fused cerebellar surface. On the mid-sagittal plane, the vermis is absent, although some vermian tissue can be preserved (partial rhombencephalosynapsis) and the primary vermian fissure and the fastigium are absent (Severino & Huisman, 2019). Neurodevelopmental outcomes of patients with rhombencephalosynapsis range from normal to severe neurological impairment. Cases with partial rhombencephalosynapsis

associated with other CNS or systemic anomalies may have abnormal neurodevelopment (Krajden Haratz et al., 2021).

1.1.3.6 Arthrogryposis multiplex congenita

Arthrogryposis multiplex congenita (AMC) is characterized by the development of joint contractures in two or more areas of the body, the most severe forms being lethal in utero or after birth. Neurogenic, mechanical and myogenic types have been described, resulting in complete or partial movement restriction and subsequent disruption of joint formation**.** Its occurrence in around 1/3,000–5,000 live births and more than 400 known syndromes are associated with arthrogryposis, making classification difficult (Hall, 2014; Hall & Kiefer, 2016).

The common background in all AMC is reduced fetal mobility, which in turn can have many causes in CNS including the brain and anterior horn cells, neuromuscular junction, peripheral nerves, muscle, connective tissue, teratogens, maternal illness, and limitation of space in utero (Hall, 2014; Hall & Kiefer, 2016). The clinical presentation is often severe when brain involvement is the cause of decreased fetal mobility and AMC.

In prenatal diagnosis it is very important establishing CNS involvement, as that usually implies a poor prognosis, and even lethality, and may affect decisions about continuing the pregnancy (Dieterich et al., 2019). Some acquired causes in CNS-linked AMC have been identified, especially congenital infections (*i.e. Zika virus, Citomegalovirus, Rubella virus*).

Among chromosomal anomalies, the most frequent cause is trisomy 18, other chromosomal aberrations include trisomies 13 and 21, recurrent translocations of chromosome 9q and 8, and microdeletions especially involving 5q23 (Dieterich et al., 2019). Congenital myotonic dystrophies are another frequent cause of fetal hypo- or akinesia presenting usually at birth with respiratory insufficiency, severe generalized hypotonia, facial diplegia and diminished deep tendon reflexes without fasciculations.

CNS involvement in congenital muscular dystrophies is associated with secondary alpha-dystroglycanopathies with a very wide range of phenotypic presentations, joint contractures appearing in the most severely affected patients (Astrea et al., 2018).

AMC could be a clinical finding also in Ponto Cerebellar Hypoplasia (PCH) type 1 and in the more severe form of PCH4, respectively linked to *TSEN54, EXOSC3, RARS2, VRK1,* and *TSEN54* pathogenic variants, PCH9 due to *AMPD2* variants and PCH12 due to *COASY* pathogenic variants. Syndromic forms of AMC and multi-organ involvement are very often associated with CNS anomalies. Some examples are Pena-Shokeir syndrome/fetal akinesia deformation sequence, Miller-Dieker syndrome, Zellweger syndrome. Peripheral and even central hypertonia has also been

observed in “channelopathies”, caused by biallelic loss of function mutations in *SLC6A9*, or heterozygous gain of function mutations of *NALCN* gene causing CLIFHADD syndrome (congenital contractures of the limbs and face, hypotonia, and developmental delay).

Pathogenic variants in some genes controlling the human cortical development have also been associated with AMC: *KIF5C, DYNC1H1, TUBB2B* in case of lissencephaly, *FLNA* and *NEDDL4* with periventricular heterotopia, and *PI4KA* and *BICD2* with polymicrogyria. More and more causes also imply involvement of both the central and the peripheral nervous systems, for example, *DYNC1H1, BICD2, ZC4H2,* or *MAGEL2* (Dieterich et al., 2019).

1.1.4 Diagnostic Imaging in Prenatal Diagnosis of CNS malformations

1.1.4.1 Ultrasound

During pregnancy, US screening for CNS malformations is carried out mainly at the time of the mid-trimester anomaly scan (Salomon et al., 2011) and relies on visualization of three axial planes, namely, the transventricular, transthalamic and transcerebellar planes; basic evaluation of the fetal spine is also part of this screening procedure, and this is performed using a combination of axial, coronal and sagittal planes.

A variety of scanning planes can be used, depending upon the position of the fetus, and whether the examination is performed transvaginally or transabdominally, proper alignment of the probe along the correct section planes usually requires gentle manipulation of the fetus, as suggested by International Society of Ultrasound in Obstetrics and Gynecology (ISUOG) guidelines (Paladini et al., 2021). It is commonly accepted that targeted fetal neurosonography has a much greater diagnostic potential than does the basic screening examination, being particularly helpful in the evaluation of complex malformations (Monteagudo et al., 2000). However, this targeted examination of the fetal CNS requires a high level of expertise that is not always available in many US facilities, since the method has not yet been implemented universally. The neurosonographic examination should include the same measurements as those commonly obtained during a basic examination: biparietal diameter, HC, atrial width of the lateral ventricles and the transverse cerebellar diameter. The anteroposterior diameter of the cisterna magna is not measured routinely; it should be measured only if there is suspicion of mega cisterna magna. Apart from the anatomic structures, fetal neurosonography should also include evaluation of the convolutions of the fetal brain, which change throughout gestation. In the last published ISUOG guidelines, the use of a 3D US approach is recommended in targeted neurosonography, particularly when a good two-dimensional image is difficult to obtain, in order to

benefit from both the enhanced resolution and the possibility of performing multiplanar imaging correlation (Paladini et al., 2021).

The midsagittal or median plane is the reference plane for assessing all major midline organs and their anomalies. Care should be taken in using CC biometry to diagnose CC hypoplasia, since a short, thin or thick CC is not necessarily synonymous of an abnormality of this anatomical structure. For this reason, a qualitative assessment is much more important than a quantitative one, checking that all four components of the CC are visible and sonographically normal. Of note, some malformations may be detectable as early as the first-trimester scan and the potential of the early anatomical assessment has increased considerably, for most CNS abnormalities. However, a follow-up neurosonographic examination after 20 GW is normally warranted except for some lethal or near-lethal anomalies, such as exencephaly-anencephaly, gross cephalocele and HPE with no need for a follow-up scan.

1.1.4.2 Magnetic Resonance Imaging

Fetal MRI is increasingly being used to further clarify findings, as a CNS abnormality is detected through US. Overall assessment of the fetus remains critical, as the brain anomaly may simply be one clue to the overall diagnosis. In this context it should be underlined that, when the indication for this complementary imaging modality is appropriate, and the diagnostic query specified clearly, MRI may contribute significantly to the final diagnosis. However, according to ISUOG guidelines, MRI should be performed only after, and to complement, a neurosonographic examination (Paladini et al., 2021). MRI becomes a particularly powerful tool when US is limited by poor acoustic windows and suboptimal sound penetration of fetus. Factors that can impede US evaluation of the uterus include large maternal body habitus, anterior placenta, oligohydramnios, difficult fetal lie, or descent of fetal head into pelvis, intervening bowel gas or other artifact-producing anatomy (Powers et al., 2022). Fetal MRI is not routinely performed before 18 GW due to the small size of the fetus and because movements usually do not allow to add any significant diagnostic information to US examination. Moreover, some structures such as CC or the cerebellar vermis are not fully developed before the 18 GW and they are therefore not adequately evaluated (Manganaro et al., 2017).

Despite MRI provides better information in late second and third trimesters, in several countries the deadline for pregnancy termination is within the 23^{th}–25^{th} GW. Therefore, fetal MRI is frequently performed before that age so to play a crucial role in terms of parent counselling and pregnancy management (Conte et al., 2016).

In some conditions, third-trimester MRI can provide unique prognostic data that add value to the usual second-trimester MRI (Powers et al., 2022). For instance, in case of apparently isolated CC

anomalies, fetal MRI performed at 18 to 22 GW should not be considered as a definitive diagnosis. In a previous work, our group suggested to perform a sequential MRI examination within 26 to 27 GW, in order to better follow the brain morphogenetic process and the pregnancy evolution (Manganaro et al., 2017). According to our experience, this is the best time-lapse to study the cortical pattern. With the later secondary sulcation and the concurrent subarachnoidal spaces decreasing, cortical abnormalities visualization could be more difficult (Manganaro et al., 2017).

In addition, Fetal MRI has the unique ability to provide high detailed anatomical information of the entire human fetus with high contrast resolution (Paladini et al., 2014). The technique has grown with the development of rapid single shot image acquisition sequences, improvement of motion correction strategies and optimized shimming techniques, among other advances. Radiologists must be familiar with the technical aspects of MR image acquisition to obtain the best information possible for optimal counseling (Cardenas et al., 2020). Image acquisition of the fetal brain must be performed in all 3 planes, the main work-horse MR sequences are T2-weighted images (Cardenas et al., 2020). Spoiled gradient echo T1-weighted images, despite their relative longer acquisition time, can detect blood products, fat, and calcium. Steady-state free precession and dynamic steady-state free precession sequences provide excellent differentiation between soft tissue and fluid interfaces, making them suitable for the evaluation of the fetal skull base and face. Echo planar imaging has the greatest sensitivity to detect blood products and can provide information about bony structures. Diffusion-weighted images are helpful to evaluate cytotoxic edema, hemorrhage, and delineate the germinal matrix (Cardenas et al., 2020).

Advances in diffusion tensor imaging have allowed improved delineation of the main white matter tracts (Jakab et al., 2017). Ongoing research includes improved delineation of fetal connectome (Ouyang et al., 2015), MR spectroscopy, and functional MR (Sanz-Cortes et al., 2015), with growing success. When possible, mother should have been fasting for at least 4 hours since low glucose levels help decreasing fetal motions and should be examined with an empty bladder to prevent urinary urge during imaging (Manganaro et al., 2017).

The patient is scanned in supine position or, if not tolerated (polyhydramnios, multiple pregnancies, late gestational age, vena cava compression, back pain), in the left lateral decubitus. No sedatives are routinely used for mother or fetus, contrast agent administration is contraindicated because gadolinium contrast agents cross the placenta and are not recommended by the US Food and Drug Administration for use in pregnancy (Moltoni et al., 2021).

At present, fetal MRI is mostly performed using a 1.5 Tesla superconducting magnet with the abdominal or cardiac phase-array coil. The safety of 3 Tesla scanning is still under debate with concerns about possible theoretical risks related to an increased amount of radiofrequency power

deposition in fetal tissue, measured in the form of specific absorption rate (SAR), and to the elevated acoustic noise intensity because of rapid gradient field switching. However, fetal MRI is currently considered a safe and effective imaging modality with no demonstrated negative effects to the developing human fetus both at 1.5 and at 3 Tesla (Moltoni et al., 2021). Contraindications are the same as for any MRI examination, and they must be excluded before scanning.
Fetal MRI adds value by increasing diagnostic certainty, allowing for detailed care and delivery planning, and providing details necessary for possible antenatal therapy (Powers et al., 2022).

1.2 Genetic Analysis in prenatal diagnosis

1.2.1 Standard Karyotype and Microarray (CGHarray and SNParray)

Genetic testing in prenatal diagnosis is a precious tool providing valuable information in clinical management and parental decision making in a complex setting. Diagnostic approaches for US anomalies are improving, and genetic testing provides valuable information about the prognosis of the fetus, being crucial to proper genetic counseling and to discuss future reproductive options. Both cytogenetics and molecular testing can be requested. Since the introduction of fetal karyotyping after amniocentesis, cytogenetic and molecular testing have evolved rapidly, and many genetic conditions can now be tested on DNA extracted from fetal samples (chorionic villus, amniotic fluid and more rarely on fetal blood) (Van den Veyver & Eng, 2015).
Standard karyotyping consists in the visualization of the entire mitotic structures, identifying chromosomal anomalies of number and structure with resolution limits of 5–10 Mb.
CMA permit the detection of even smaller chromosomal imbalances, called CNVs, reaching a resolution of 50-75 Kb. The main approaches used are array-comparative genomic hybridization (array-CGH) and single nucleotide polymorphism array (SNP-array).
Array-CGH is one of the most widely used CMA techniques and compares DNA content from two differentially labeled genomes with a resolution limited only by the size of the DNA probes immobilized in the array and the natural distance between these sequences located on the chromosome. Conversely, SNP-array analyzes the sample through hybridization with allele-specific oligonucleotide probes, additionally identifying runs of homozygosity (ROHs), which can be caused by consanguinity or uniparental disomy, and mosaicism at a lower percentage than a-CGH (Mastromoro et al., 2022). Karyotype anomalies are found in 8-10% of US structural abnormalities (Levy & Wapner, 2018). Several studies have investigated the use of CMA in fetuses presenting abnormal US findings, showing a further 6-7% diagnostic yield over karyotype (Lovrecic et al., 2016; Levy & Wapner, 2018).

Fewer studies have explicitly reported on detection rates focused on CNS anomalies. The prevalence of pathogenic CNVs in these studies over karyotype varies between 3.7% and 10.9% (Sun et al., 2015; Schumann et al., 2016; Krutzke et al., 2016).

Recently, the prevalence of pathogenic CNVs and Variants of Uncertain Significance (VUSs) detected by CGH-Array in a cohort of 238 fetuses with different CNS anomalies was investigated (Santirocco et al., 2021), showing a similar prevalence of 7% for CNVs and 8% for VUSs, once aneuploidies of chromosomes 13, 18, 21, X, and Y were excluded.

The prevalence of pathogenic CNVs was higher when other structural abnormalities were present (18.4% of cases with an associated anomaly *versus* 3.8% of cases of isolated anomalies) (Santirocco et al., 2021). The highest prevalence of pathogenic CNVs in this study was found for PF anomalies (12.9%) being cerebellar hypoplasia the most prevalent (33%), followed by mega cisterna magna (20%), moderate ventriculomegaly (11%) and spina bifida (3.7%) (Santirocco et al., 2021).

1.2.2 Next Generation Sequencing (NGS) technology

Next Generation Sequencing (NGS) has revolutionized clinical practice in Medical Genetics, identifying point mutations and small indel within a limited timeframe and becoming a staple of prenatal diagnosis (Wou et al., 2019).

The term NGS refers to a group of massive and parallel nucleic acid sequencing technologies. The techniques currently used in clinical practice are Second Generation Sequencing, or Short Read Sequencing, approaches (Goodwin & McPherson, 2016). The target region sequence is inferred by aligning multiple short individual partially overlapping reads to a reference sequence (Goodwin et al., 2016). NGS approaches enable the sequencing of targeted gene panels or broader fractions of the genome with rapid turn-around time (TAT), thus NGS applications are represented by targeted panels, ES, and genome sequencing (GS) (Sun et al., 2015).

NGS experiment workflow is resumed in Fig.1 (Guadagnolo et al., 2021). Genomic DNA extracted from the samples is first processed in libraries. Purified genomic DNA is fragmented, then the fragments go through an end-repair phase and barcode and adapters ligation. Barcodes are unique sequences identifying specific samples within a run. Adapters are universal sequences used for PCR amplification and fragment capture. Some protocols require targeted enrichment (with PCR or biotin-streptavidin magnetic pulldown) to capture the desired fraction of the genome. The libraries then undergo the crucial step of clonal cluster PCR amplification. This step leads to thousands of identical copies of the original DNA fragments in defined areas, allowing spatial resolution of localized, non-overlapping clonal clusters. This can be done with either emulsion-PCR, on hydrophilic beads emulsified in oil, or Solid-state bridge PCR, in the micro-wells of a patterned flow cell.

The sequencing reaction can be based on DNA ligase (Sequencing by ligation) or DNA synthetase (Sequencing by synthesis). The latter is the most common. Sequencing by synthesis can take place by Single Nucleotide Addition or by Cyclic Reversible Termination (Guadagnolo et al., 2021). (Fig.1).

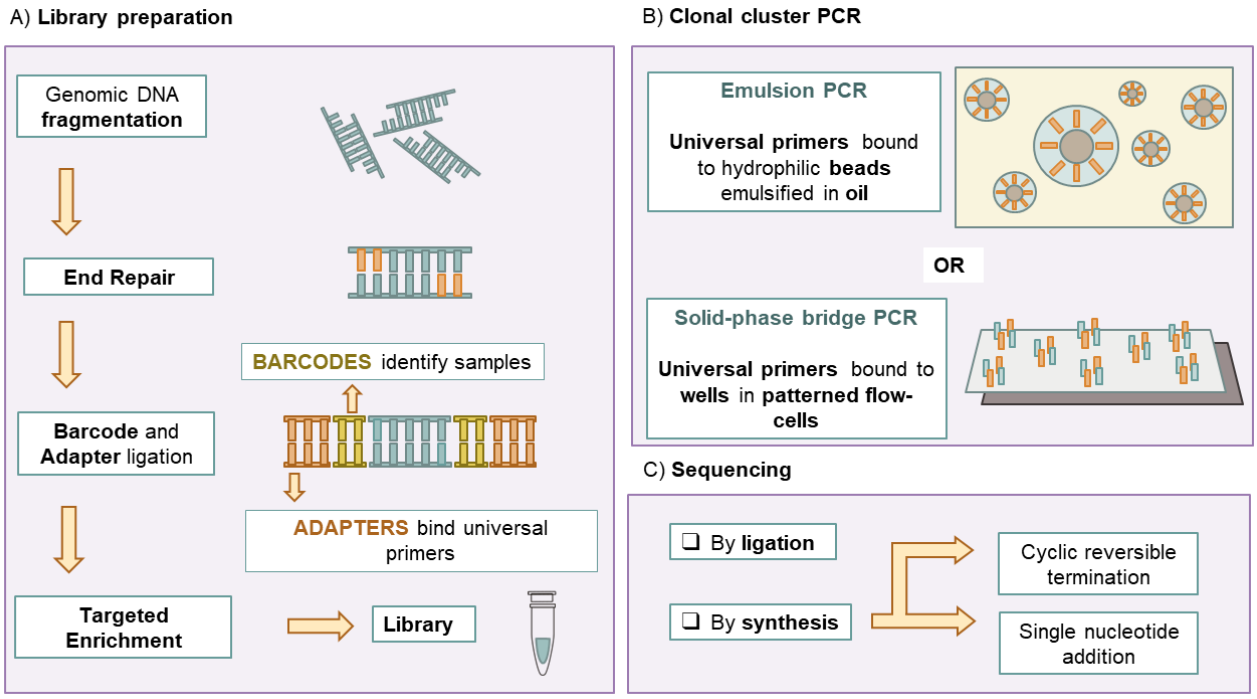

Fig.1 NGS workflow from Guadagnolo et al., 2021

NGS bioinformatic workflow is resumed in Fig.2. The sequencing reaction generates millions of short reads (40-to-400 nucleotide long). The reads are processed by removing duplicates, and barcode and adapter sequences. Individual reads are retained in a FASTQ file. They are then aligned to the reference genome, generating a BAM file. Variants are identified (called) from nucleotide positions differing from the reference sequence, and gathered in a VCF file, consisting in a list of genomic coordinates with the reference sequences, the putative variants and quality scores.

These variants are then annotated with information gathered from various databases, on variant frequency, gene(s) involved, gene products, predicted deleteriousness, reported pathogenicity or benignity. They are then manually analyzed by filtering and prioritization. Variants with no impact on gene products, high frequency in the general population and not segregating with the phenotype are ruled out. The remaining variants are prioritized by various criteria, for example by potential relation to the phenotype or predicted deleteriousness (Guadagnolo et al., 2021) (Fig.2).

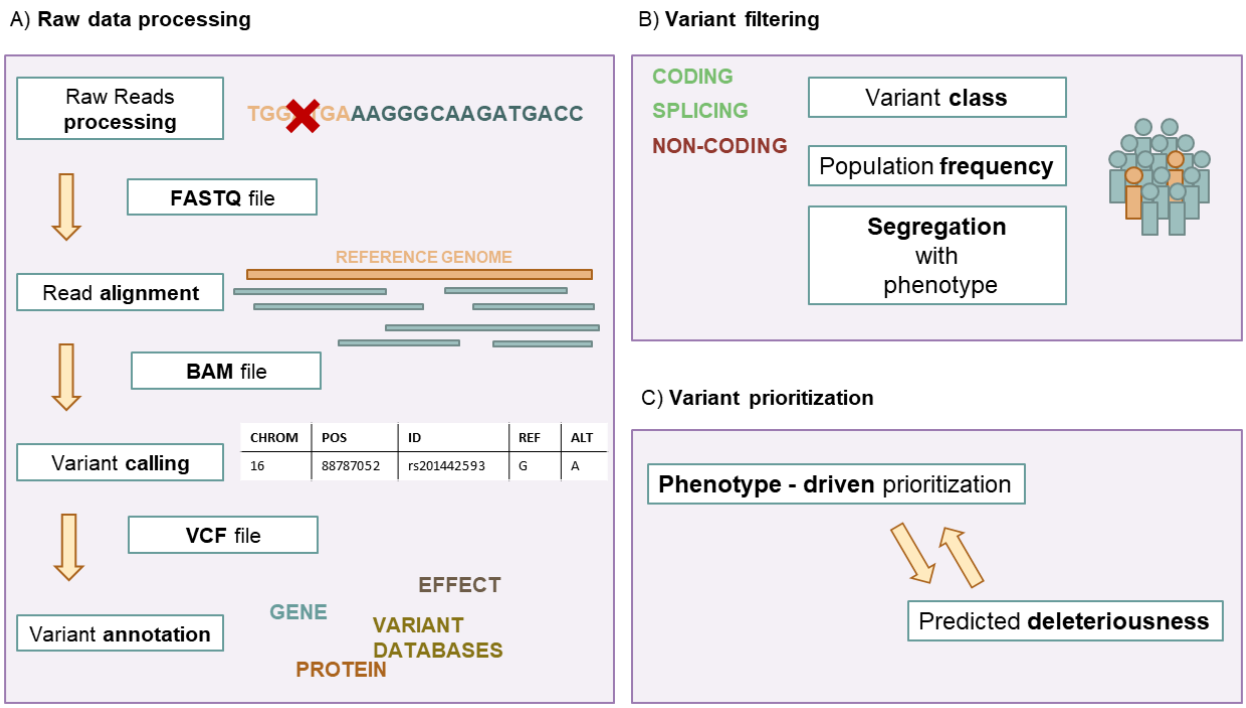

Fig.2 NGS bioinformatic workflow from Guadagnolo et al., 2021

A key analytical parameter assessing the reliability of NGS data is coverage. Depth of coverage is the number of individual reads including a given nucleotide. Average depth/coverage is the average number of reads per position. Breadth of coverage is the targeted sequences percentage covered at a given depth (Sims et al., 2014). Despite their undeniable strengths, NGS technologies do have challenges. Especially in capture-based methods, some regions may not be appropriately covered (Pengelly et al., 2020). Unbalanced rearrangements are difficult to assess (Moreno-Cabrera et al., 2020), single-exon deletions or duplications and CNV analysis, require dedicated algorithms and software, not all widely available (Moreno-Cabrera et al., 2020).

1.2.3 NGS approaches: ES, CES, WES, GS

Genome-wide DNA sequencing, which includes both ES and GS, focuses on finding disease-causing variants in the genome. Presently, ES, which evaluates the coding sequence of human genes, comprising 1.5%–2% of the genome, is a well-established tool in the diagnosis of pediatric and adult genetic disease. When the test is designed to ideally capture the set of all coding exons, it can also be called Whole Exome Sequencing (WES) (Goodwin & McPherson, 2016).

The rationale behind this is that 85% of known disease-causing mutations are exonic and Clinical Exome Sequencing (CES) or Targeted/Focused Exome Sequencing or Medical Exome Sequencing, captures genes implied in Mendelian disorders (Pengelly et al., 2020).

This set of 5000-7000 genes, also called "Mendeliome", are causatively associated with known single gene disorders catalogued in OMIM (https://www.omim.org), and represent a dynamic entity, as research is still evolving (Pengelly et al., 2020).

GS refers to the unbiased sequencing of the genome, without targeted capture (Goodwin & McPherson, 2016).

ES is not the ideal approach for the identification of CNVs, due to the non-contiguous nature of the captured exons and to the extension of most CNVs beyond the regions covered by the exome kit (Belkadi et al., 2015). In fact, batches of samples processed in a similar fashion are needed for the analysis and the coverage of sequencing data needs to be homogeneous within and among samples, to allow differentiation between potential gain or loss of copies, and the technical variability of sequencing. The sequencing coverage is particularly important to distinguish true heterozygous deletions from low-covered regions. This is a known issue particularly for the first exons of genes and for genes and exons in low-mappability regions (Royer-Bertrand et al., 2021). However, in the last years, ES has greatly progressed with libraries targeting exons of all genes simultaneously and with improved coverage and uniformity, and numerous methods have been developed to detect CNVs from ES data and there are also kits able to enrich for CNVs. These advancements, in combination with bioinformatics software improvements, have allowed direct CNV detection from ES raw data to become more and more precise (Zhou et al., 2021). Based on the coverage (reads-depth) of one patient compared to a set of patients sequenced at the same time and/or sequenced using the same conditions, NGS-based CNV software can detect regions with significantly more reads (gain of copies) or fewer reads (loss of copies) than the average (Royer-Bertrand et al., 2021).

In this view, GS yields the highest exon coverage, while also providing information on non-coding sequences and CNV imbalances (Barbitoff et al., 2020). However, we are far from understanding most of the non-coding variants and the bulk of sequencing data and bioinformatic analysis hinders a widespread GS application. When performing GS or ES, the analysis can be initially limited to a given subset of genes, an *in silico* panel.

Currently, ES is extensively performed in post-natal practice, with a diagnostic yield of 25-45% (Van den Veyver, 2016; Seo et al., 2020). This has led to some professional societies supporting its use as a first-line test in children and adults with developmental and intellectual disabilities.

New data indicate that in these populations, GS, which analyzes most of the 3 billion base pairs on the genome, has equal to superior diagnostic rate compared to exome sequencing, but has a higher incidence of VUSs and possibly incidental and secondary findings (IF and SF, respectively) (Malinoski et al., 2020; Manickam et al., 2021).

Diagnostic prenatal ES (pES) was initially performed as second- or third-line testing in fetuses with structural anomalies, non-diagnostic karyotype and CMA, familial history and/or prenatal presentation suggesting monogenic disorders (Mone et al., 2018; Krstic & Obican, 2020).

Proper indications for pES are still debated, as well as the choice among WES, CES or other approaches. Presently, pES is the predominant approach used for genome-wide sequencing in prenatal clinical practice (Mastromoro et al., 2022) and it is extremely helpful in elucidating and expanding fetal phenotypes of known disease-causing genes. Multiple studies, small case series, and case reports, have illustrated the value of pES, but it is important to recognize the differences between these studies and the study- specific sequencing and interpretive approaches (see Table 1, from Van den Veyver et al., 2022).

Method	What is sequenced?	What is analyzed?	Depth	Advantage	Disadvantage	Comment
(Whole) genome	~95% of 3 billion bp (introns, exons, non-coding)	~95% of 3 billion bp (introns, exons, non-coding)	30–40 X	Comprehensive; NC genome data; Potential for SV, CNV, aneuploidy detection	More VUS/GUS; possible more SF/IF; limited data on SV and NC-V interpretation/disease association; expensive; lower coverage	Research only
Capture (whole) exome	Exons and flanking intron sequence of ~20,000 genes (1.5% of genome)	Exons and flanking intron sequence of ~20,000 genes (1.5% of genome)	~100 X	All sequenceable exons analyzed Higher coverage	Only coding sequences, not all genes equally captured; more risk for GUS/VUS. SF, IF than clinical, prenatal phenotype and phenotype-driven panels.	Acceptable clinical option with MDT expert involvement
Digital (whole) exome	~95% of 3 billion bp (introns, exons, non-coding)	Exons and flanking sequence of ~20,000 genes (1.5% of genome)	30–40 X	All sequenceable exons analyzed; easily adaptable analysis pipeline if new gene discovery	Same as above; lower coverage than capture (whole) exome	Research only
(Digital) prenatal phenotype panel	Exons and flanking intron sequence of ~4000 genes	Exons and flanking intron sequence of ~1300 genes	~100 X	Comprehensive for known genes with prenatal phenotypes; lower risk for GUS/VUS than whole/clinical exome; high coverage: may include method for CNV detection; reanalysis possible. SFs not detected. Infrequent IFs	Only coding sequences of known genes for prenatal phenotypes not all genes equally captured; regular panel update required.	Acceptable clinical option with MDT expert involvement
(Digital) phenotype-driven gene panel	Exons and flanking intron sequence of ~4000 genes	Exons and flanking intron sequence of few to 100's of genes	~100 X	Known genes for specific syndrome/ organ phenotype: Lower risk for GUS/VUS than whole/clinical/ prenatal exome; higher coverage; may include method for CNV detection; reanalysis possible. SFs not detected. Infrequent IFs	Only coding sequences of known genes for prenatal phenotypes not all genes equally captured; regular panel update required.	Acceptable clinical option with MDT expert involvement
Clinical/Medical exome capture	Exons and flanking intron sequence of ~4000 genes	Exons and flanking intron sequence of ~4000 genes	~100 X	Known disease genes; includes childhood disorders w/o prenatal phenotype; lower risk for GUS than whole exome; higher coverage	Only coding sequences of known disease genes, not all genes equally captured; more risk for GUS/VUS and SF/IF than prenatal phenotype and phenotype-driven panels; can become outdated with new disease gene discovery	Acceptable clinical option with MDT expert involvement
Gene panel capture	Exons and flanking intron sequence of few to 100's of genes	Exons and flanking intron sequence of few to 100's of genes	~100 X	Known genes for specific syndrome/ organ phenotype: Lower risk for GUS/VUS than whole/clinical/ prenatal exome; higher coverage; may include method for CNV detection. SFs not detected. Infrequent IFs	Only coding sequences of known genes for specific phenotypes; can become more quickly outdated with new disease gene discovery; panels usually designed based on postnatal presentations	Not preferred, but acceptable option if exome not available

Abbreviations: CNV, copy number variants; GUS, genes of uncertain significance; IF, incidental findings; MDT, multidisciplinary team with genetics expertise; NC, non-coding; SF, secondary (actionable) findings; SV, structural variants; VUS, variants of uncertain significance.

Table 1 Summary of different sequencing approaches from Van den Veyver, 2022

Some include sequencing and interpretation of variants in the exons of nearly all genes, while others focus on the clinical exome, or alternatively, on the ~1600 genes clearly associated with genetic conditions known to present with malformations detectable in the fetus or neonate (Mastromoro et al., 2022). Furthermore, small series of prenatal genome sequencing (pGS) are now emerging and there are ongoing trials evaluating pGS (Wang et al., 2022).

Recently, pGS was applied on a prospective cohort of 37 singleton fetuses with US-identified structural abnormalities undergoing invasive prenatal testing. Diagnostic variants were identified in 5 fetuses (14%), and a further 7 fetuses (19%) had VUSs that may explain the phenotypes. Importantly, pGS also identified all pathogenic variants reported by CMA (2 CNVs, 5%).

In this cohort pGS allowed to reach diagnoses (SNVs and CNVs) in 19% of fetuses with structural anomalies, demonstrating the potential of replacing multiple consecutive tests, including CMA, gene panels, and pES, and to provide the most comprehensive analysis in a timely manner necessary for prenatal diagnosis (Wang et al., 2022). GS also provides a higher resolution for CNV detection compared to CMA (Fig.3) (Liu & Vossaert, 2022).

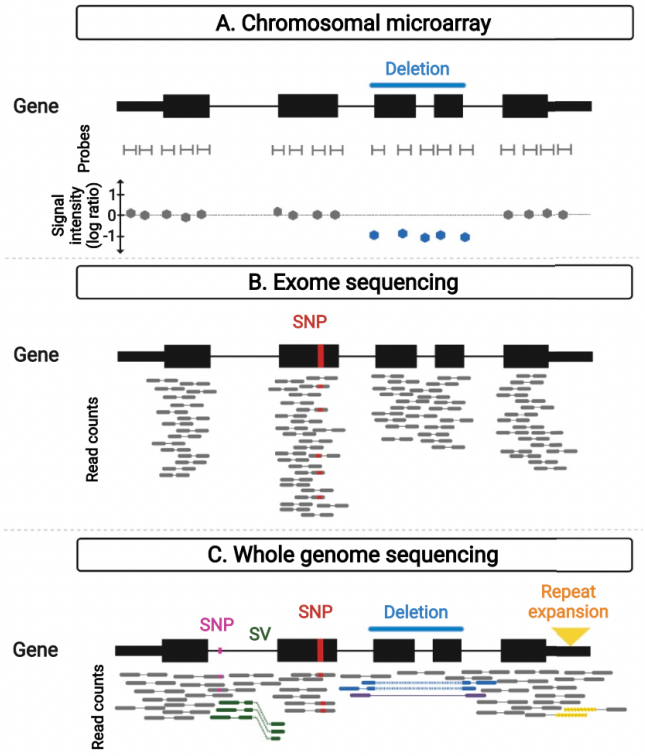

Fig.3 Comparison of variant detection for different variant types between CMA, ES and GS from Liu and Vossaert, 2022. (A) CMA is capable of CNV detection, such as a heterozygous deletion (indicated in blue). The detection of the deletion depends on the coverage and signal from probes laid out on the design of the microarray. A sufficient number of probes located within the genomic region of the CNV is necessary for robust detection of the variant. (B) Small variants such as SNPs or INDELs can be detected in coding regions from an ES assay. Variants located in noncoding regions are not captured and therefore will not be detected. CNV can in theory be detected by analysis of the ES read count data; however, the robustness of such an analysis is limited. This challenge is illustrated in the figure by the unevenness of read depth (the number of reads covering each base averaged across a region) over the gene, which can potentially mask read depth alterations representing a real copy number variant. (C) GS generates data that can support variant calling of coding and noncoding small variants, CNVs, structural variants (SV), and repeat expansions. CNVs can be more readily detected in GS compares to ES because of the more even baseline sequencing

depth. CNV calling is boosted by the higher probability to detect breakpoints by the split-read algorithm (as illustrated by the two broken sequencing reads connected by the blue dotted lines) and the read-pair algorithm (the purple read pairs that are spaced further away compared to other read pairs) in the figure. In addition, the green split-read pairs illustrate a SV such as a translocation, with part of the read aligning to a location elsewhere in the genome. The yellow reads highlight the possibility to detect alleles with expanded repeat units from whole genome sequencing data.

The raw data from GS carry read count information at each of the three billion bases in the human genome; in contrast, CMA is physically limited in its data content because a maximum of only one to four million oligonucleotide probes can be printed on a microarray slide. Thus, it is possible for GS to detect CNV missed on a CMA because either the microarray does not have adequate oligo density to support a sensitive detection for small CNVs, or the genomic region of interest is not interrogated well during the array design because other genomic loci were prioritized (Liu & Vossaert, 2022). Moreover, CNV calling from GS can be achieved beyond the conventional *"counting paradigm"* as is done in microarrays: precise or estimated breakpoints of genomic rearrangements can be retrieved from GS data (Fig.3) (Gross et al., 2019) as well as low-level mosaic changes. Obviously, balanced copy number neutral structural variants will not be revealed by CMA, but can be captured by WGS (Liu & Vossaert, 2022).

Given this variability on application, the diagnostic yield for pES still varies widely among studies (Guadagnolo et al., 2021). Recently, we performed a meta-analysis to evaluate the diagnostic yield and the rates of VUSs in fetuses undergoing molecular testing (CMA, ES, GS) due to US anomalies (Mastromoro et al., 2022). Prenatal CMA and ES cohorts deemed eligible for quantitative analysis were divided into two groups for each approach: Group A, including cohorts with pooled different indications, and Group B, including fetuses enrolled for a specific single-district anomaly. In this meta-analysis in Group A, ES showed an overall diagnostic rate of 19.47% C.I. (18.94–20.01%).

We also stratified the diagnostic yields by single-district anomalies retrieved from Group A and B. However, the classification of the anomalies and their associations had little consistency among studies, confounding the ability to obtain specific diagnostic rates per organ system. Data for the meta-analysis of the specific organ anomalies was not available for all the papers included in Group A, and it was not performed for CNS anomalies in Group B. When data were available, most papers included in the anomaly-specific subcategories both fetuses with isolated features and associated anomalies, without the possibility to discern the two categories. For some of them with more than one anomaly, the count was repeated in several subcategories with variable inclusion criteria. The pooled diagnostic rate was 14.77% C.I. (13.23–16.32%) in the group of any cardiovascular anomaly, 34.11% C.I.(31.42–36.80%) in musculoskeletal anomalies, 21.23% C.I.(17.49–24.97%) in genitourinary anomalies, 34.88% C.I. (30.21–39.55%) in craniofacial anomalies, 24.70% C.I. (23.94–25.45%) in CNS anomalies and 10.53% C.I.(7.53–13.52%) in abdomen or body wall anomalies.

The subcategory of isolated NT when data were retrievable (6/17 papers) showed a diagnostic rate of 13.46% C.I.(10.19–16.74%). The diagnostic rate in the group of any dynamic anomalies was 20.78% C.I. (18.71–22.84%) (Mastromoro et al., 2022).

Although for some categories more evidence is needed to refine numbers, there are now sufficient data to begin differentiating diagnostic yields by specific organ system or number of organ systems affected, for this reason, during my PhD course, we decided to perform a meta-analysis only focused on fetuses affected by apparently isolated and non-isolated CNS anomalies, undergoing pES. Methods and result of this meta-analysis will be illustrated in the methods and result section. Nowadays, the routine use of pES or pGS as a diagnostic test on all pregnancies cannot currently be supported due to insufficient validation data and knowledge about its benefits and pitfalls, however in literature some papers describing their use in clinical practice are available.

In 2022, Vaknin et al., performed pES in 353 pregnancies (Vaknin et al., 2022), in Group 1 including 143 pregnancies with high clinical suspicion for a genetic disease and in Group 2 including 210 pregnancies with no notable abnormal fetal ultrasound findings (subcategories 2a: low risk pregnancies with minor ultrasound findings and 2b: normal pregnancy surveillance). Overall, 26 (7.36%) fetal analyses had pathogenic (P)/likely pathogenic (LP) variants. In group 1, 20/143 (13.99%) cases had P/LP variants. In group 2, 6/210 (2.86%) cases were found to have P/LP variants [5/50 in (2a) and 1/160 in (2b)]. Fetuses in group 2a were referred for: 1) HC -1.70 SD at 22 GW (fetus resulted compound heterozygous for LP and P *ERCC2* variants); HC <3 centile was found at 25 GW considering the genotype matching the phenotype; 2) absent ductus venous, disclosing a de novo LP *CASK* variant as an incidental finding; 3) double ductus venosum, disclosing a LP heterozygous *MAGEL2* variant as an incidental finding; 4) echogenic colon without dilated intestine and normal peristalsis, disclosing a de novo heterozygous *POGZ* variant and considering the genotype matching the phenotype; 5) vertical pocket 11 mm and mild decreased in fetal movements, disclosing a de novo heterozygous *TLK2* variant considering genotype matching the phenotype; 6) no indication, pES was required to the couple' wishes, disclosing a P de novo heterozygous *KCNK4* variant, as an incidental finding. In conclusion this preliminary and very recent study showed an unexpected rate of abnormal findings on pES even in apparently normal pregnancies (Vaknin et al., 2022).

1.2.4 Prenatal genome-wide sequencing guidelines

In 2018, the International Society for Prenatal Diagnosis (ISPD), joined by the Society for Maternal Fetal Medicine (SMFM), and the Perinatal Quality Foundation (PQF) published the first Position Statement specifically on the use of genome-wide sequencing for the prenatal diagnostic work-up of pregnancies complicated by fetal structural anomalies (ISPD, SMFM, PQF, 2018). At that time, this

was an emerging genomic diagnostic method being evaluated in a limited number of centers. Since then, both its research and clinical use have rapidly increased (Mastromoro et al., 2022). This has led to the development of local guidance or "points to consider" statements by national professional societies in some countries, but for many parts of the world, such guidance is still lacking. Surprisingly, in the last 5 years, there were no worldwide guidelines for pES application, despite its increasing importance. Recently, an Updated Position Statement of ISPD on prenatal genome-wide sequencing has been published (Van den Veyver et al., 2022), recommending important points for consideration by those who perform pES or pGS and report results, including those who counsel and obtain informed consent from parents.

The major points to consider are hereinafter fully reported from this statement, being officially published in March 2022 (Van den Veyver et al., 2022):

- *Diagnostic sequencing for fetal indications is best done as a trio analysis,* for a *timeliness of result interpretation and assignment of pathogenicity for detected sequence variants. If only proband sequencing is performed, validation of diagnostic or potentially diagnostic findings best includes a determination of inheritance through targeted testing of samples from biological parents.*

- The provider or providers, who offer sequencing for fetal indications and who conduct the pre-test education and counseling, obtain informed consent, and conduct post-test counseling and result disclosure must have an in-depth understanding of the benefits and risks to the fetus and parents of trio-based sequencing. This is typically within the domain of a genetic health provider, or a relevant other specialist with extensive genetic training. Interpretation of results and post-test counseling are highly complex and are best conducted in consultation with a multidisciplinary team with expertise and experience in both the clinical and laboratory aspects of prenatal diagnosis and fetal sequencing. Ideally, members of the team will have access to pertinent clinical records, sequencing results, and fetal imaging studies.

- Expert pre-test patient education, counseling and informed consent, as well as post-test counseling are essential. It is recommended that the following minimal elements be considered:

a. Pre-test education and counseling should be individualized and offered to both parents when possible.

b. Counseling requires communicating detailed and often complex genetic information in a manner that balances the reality of variable genetic literacy and time constraints. Patient counseling, both consistency and knowledge, is aided by educational tools.

c. As diagnostic sequencing can reveal genetic information about the fetus that can impact one or both parents and the family unit, ideally if possible, both biological parents should be involved in the consent process as follows:

i. If trio sequencing is undertaken, each parent should provide separate informed consent for the sequencing of his or her own sample.

ii. As for all prenatal procedures, the pregnant woman alone can provide consent for the invasive procedure that is performed on her to obtain the fetal genetic material.

iii. The pregnant woman can provide consent for the fetal genetic assessment (in a duo sequencing approach) if the biologic father is uninvolved as a decision maker, or cannot be contacted.

d. Pre-test counseling and informed consent must address the following for each genome analyzed (i.e., the fetus and each biological parent) and should reflect the sequencing analysis and reporting policy of their local testing laboratory.

- *i. The types of results to be conveyed (variants that are pathogenic, likely pathogenic, of uncertain significance, likely benign, and benign). The approach to reporting variants of uncertain significance should be disclosed during pretest counseling and included in consenting.*
- *ii. Realistic expectations about the chance that a clinically significant result will be obtained. The understanding that even with non-diagnostic result an underlying genetic disorder may still be possible.*
- *iii. The timeframe (range) when a result can be expected.*
- *iv. The possibility that no result is obtained (e.g., related to sample quality), or that a result may not be available in a timely fashion to influence pregnancy or neonatal management.*

e. The importance of data sharing in de-identified databases is crucial for genetic health care. Where this is available, consent should be obtained for storing this data and parents should be advised of who will have access and for what purpose.

f. It is recommended that all individuals undergoing sequencing always receive post-test counseling, including those for whom sequencing has not yielded clinically useful information. Such counseling should be provided by individuals with relevant genetic expertise.

i. *Post-test counseling and return of results should take into account the documented patient and provider pre-test discussions of options and choices, including about which results will be returned.*

ii. *Result disclosure and post-test counseling will be based on knowledge that is current at the time of result interpretation and disclosure.*

iii. *Potential changes over time are likely to occur in our knowledge of disease genes, pathogenicity of sequence variants and fetal phenotypes.*

    1. *This may result in reclassification either upward or downward of identified variants.*

    2. *This should include information on available strategies for sample and/or data storage, and re-analysis of uninformative sequencing analysis.*

    3. *Reanalysis should be considered as an option if indicated clinically, for example, if additional phenotype information is available from the proband after birth or during development, or if a future pregnancy is planned. Parents should be made aware of this possibility at post-test counselling and know how to contact their genetic health provider in these eventualities.*

iv. *Results disclosure should include a discussion regarding the future implications for the parents' reproductive and testing options.*

v. *Parents should be given written information about the results, the genetic counseling, implications for family members and their reproductive options in a language appropriate for non-experts, in a format that is easily accessible for future reproductive decisions.*

The statement also indicates current clinical indications for clinical use:

1. The current existing data support that prenatal sequencing is beneficial for the following indications:

a. A current pregnancy with a fetus having a major single anomaly or multiple organ system anomalies:

i. *For which no genetic diagnosis was found after CMA and a clinical genetic expert review considers the phenotype suggestive of a possible genetic etiology.*

ii. *For which the multiple anomaly "pattern" strongly suggests a single gene disorder with no prior genetic testing. As pES is not currently validated to detect all CNVs, CMA should be run before or in parallel with pES in this scenario.*

b. A personal (maternal or paternal) history of a prior undiagnosed fetus (or child) affected with a major single or multiple anomalies:

i. *With a recurrence of similar anomalies in the current pregnancy without a genetic diagnosis after karyotype or CMA for the current or prior undiagnosed pregnancy*.

Point a.i. above also applies in these circumstances.

ii. When such parents present for preconception counseling and no sample is available from the affected proband, or if a fetal sample cannot be obtained in an ongoing pregnancy, it is considered appropriate to offer sequencing for both biological parents to look for shared carrier status for autosomal recessive mutations that might explain the fetal phenotype. However, where possible, obtaining tissue from a previous abnormal fetus or child for pES is preferable.

There is currently no evidence that supports routine testing (including upon parental request) on fetal tissue obtained from an invasive prenatal procedure (amniocentesis, CVS, cordocentesis, other) for indications other than fetal anomalies:

a. There may be special settings when prenatal sequencing in the absence of a fetal phenotype visible on prenatal imaging can be considered, such as with a strong family history of a recurrent childhood-onset severe genetic condition with no prenatal phenotype in previous children for whom no genetic evaluation was done and is not possible. Such scenarios should be reviewed by an expert multidisciplinary team preferentially in the context of a research protocol. If sequencing is done for this indication, it must be done as trio sequencing, using an appropriate analytical approach.

Finally, also laboratory recommendations, pertaining to quality standards, variant interpretation and the return of results were detailed in the document, specifying that accredited diagnostic laboratories with relevant experience in prenatal genomic testing and interpretation should fully validate technical workflows and bioinformatic pipelines and clearly communicate them in the laboratory report:

- *Clinical information about the phenotype is an integral component of interpretation of sequencing data. Before testing is initiated, clinical information must be submitted by the referring clinician. This should include details of family and parental medical histories and details of all prenatal imaging (ultrasound, MRI, etc.) performed. The imaging reports should include details of fetal biometry. Whilst use of human phenotype ontology terms is preferred, development of these terms as pertains to the fetus is still in development. In some circumstances, review of images may be helpful.*

Laboratories are encouraged to set up systems to facilitate submission of standardized phenotype information as part of the test requisition process.

- Initial variant annotation and filtering is best performed by the diagnostic laboratory. A clear variant filtering strategy should be employed and made known to the referring clinician and stated on the laboratory report. When a trio has been sequenced, in- heritance filtering can be used. However, if no candidate variant is found by this approach it is recommended to analyze variants without taking into account assumed inheritance patterns, such that for example, de novo variants can be uncovered. This can be done by comparison of identified variants to those listed in databases of known genes associated with genetic disease, as well as those that contain variants identified in healthy people, such as ClinVar, Human Gene Mutation Database (HGMD) (https://www.ncbi.nlm.nih.gov/clinvar/, https://www.hgmd.cf.ac.uk/ac/index.php).

- Variant classification is also best performed initially by the diagnostic laboratory according to local best practice guidelines such as American College of Medical Genetics and Genomics (ACMG). Interpretation of pathogenicity and attributed clinical significance should be informed by the fetal phenotype and other relevant clinical information. We therefore recommend that variants of interest are discussed using a multidisciplinary team approach that includes clinical scientists, specialists in imaging, clinical geneticists or genetic counselors with prenatal expertise, as well as experts in prenatal diagnosis in order to take into ac- count all relevant clinical information.

- Considering the complexity of sequencing data, dialogue between laboratories and referring clinicians, with support of relevant clinical experts for final interpretation or possible revision of interpretation is highly recommended.

- Result reporting from sequencing data on fetal samples is best focused on pathogenic and likely pathogenic variants in genes that are relevant to the fetal phenotype.

- It is recognized that some laboratories may report variants of uncertain significance in strong candidate disease genes for the fetal phenotype, for example, in an autosomal recessive gene which is relevant for the fetal phenotype, when a pathogenic (or likely pathogenic) variant is inherited from one parent along with a variant of uncertain significance from the other parent. This should be addressed in pre-test counselling, and in these situations, expert genetic post-test counselling is highly recommended.

1.2.5 Prenatal genome-wide sequencing challenges

Incidental and secondary findings, and VUSs are significant challenges for laboratories and clinicians, using prenatal genome-wide sequencing (Westerfield et al., 2014; Horn & Parker, 2018). IF are non-actionable findings independent from clinical indication. SF are variants from a specific ACMG gene list, unrelated to the primary presentation and medically actionable. ACMG previously published guidance for reporting SF in the context of clinical ES and GS in 2013, 2015, 2017, and 2021 (Green et al., 2013; ACMG Board of Directors, 2015; Kalia et al., 2017;). The first ACMG statement (56 genes) recommended to include children, explicitly excluding fetuses (Green et al., 2013). The subsequent revised document in 2015, suggested to offer an opt-out option after pre-test counseling (ACMG Board of Directors, 2015). The 2020 ACMG statement focused only on points to consider for prenatal sequencing, confirming the opt-out option, and recommending reporting secondary findings if parents consented (Monaghan et al., 2020). In 2022, un update of the ACMG gene list (version v.3.1) was published, increasing the number of genes included (78 genes) (Miller et al., 2022). This update does not provide specific indications for SF and IF prenatal reporting, stating that "clinicians should apply their own professional judgment to the specific clinical circumstances presented by the individual patient or specimen" (Miller et al., 2022). These findings can hamper genetic counseling, especially in prenatal diagnosis (Westerfield et al., 2014; Horn & Parker, 2018). In fact, in prenatal diagnosis, there is no universal consensus on the management of IF and SF and each center should convey their policy detailing whether they are or are not reported, and if reported what is included for parents and fetus (Van den Veyver et al., 2022). Currently, for IF, ACMG recommends reporting only high-penetrant pathogenic variants known to cause moderate/severe childhood onset disorders (Monaghan et al., 2020). ACMG statement also recommends laboratories to establish clear policies regarding whether IF report is limited to fetal variants or parents are also included (irrespective of fetal inheritance). Of note, the laboratory's analysis process could be set to limit the assessment of parental variants to only those present in the fetus (Monaghan et al., 2020). Hereinafter, the last available recommendation published in March 2022 in the Updated Position Statement of ISPD are fully reported (Van den Veyver et al., 2022):

1. Secondary findings: where appropriate, the option for inclusion or exclusion of SF in the fetal and parental sequence should be addressed. SF are pathogenic and likely pathogenic variants in a defined set of genes that are medically actionable, that is, cause disorders for which a healthcare intervention can improve outcome in asymptomatic individuals (i.e., they are medically actionable and include for example cancer susceptibility genes) and if disclosed, the implications for other family members.

a. Parental SF: Each parent should consent separately to inclusion or exclusion of SF for their own sequencing results.

b. Fetal SF: Fetal SF with a moderate to severe childhood condition should be discussed for inclusion/exclusion consent.

c. If using a panel approach to analysis parents should be advised that SFs are not looked for.

2. Incidental findings: where appropriate, the option for inclusion or exclusion of IF should be addressed. IF are pathogenic or likely pathogenic variants in genes not related to the testing indication, and not in the defined SF list of medically actionable genes. These are variants that cause late-onset conditions including neurological, neuromuscular, cardiovascular, or inherited cancer syndromes, and if disclosed, have implications for other family members. They can also include genetic carrier states (autosomal recessive, dominant and X-linked) which should be considered separately

a. Parental IF: Each parent should consent to inclusion or exclusion separately

b. Fetal IF: Fetal IF should be discussed for inclusion/ exclusion during consent. These include IFs in genes associated with neurodevelopmental disorders, intellectual disability, or metabolic conditions that are highly penetrant and are known to cause moderate to severe childhood disorders. These conditions may present without ultrasound findings.

c. If using a panel approach to analysis parents should be advised that the risk of detecting IFs is small and will be restricted to genes on the panel used.

Finally, in prenatal diagnosis, the possibility of uncovering non-paternity or close parentage (e.g., consanguinity or an incestuous relationship between the biological parents of the fetus) should be discussed. Pretest counseling should include how a specimen from a nonbiological parent will be analyzed and reported. Inconclusive results could be also linked to a variant in a candidate gene (gene with limited evidence of disease association). The choice of reporting this kind of variant is currently ascribed to the single laboratory policy. ACMG statement suggests the classification of a variant in a candidate gene no higher than a VUS and recommends to clearly specify the policy during the consent process (Monaghan et al., 2020). In pES (and pGS), the likelihood of detecting a VUS increases and the question of whether a VUS should be reported or not has not been resolved. Standardized guidelines are not available, resulting in inhomogeneous conducts, even within the same country. In the last "Points to consider document" (Monaghan et al., 2020), ACMG highlights how laboratories currently have different approaches to report a VUS prenatally, rather recommending defining clear

policies. The first point to clarify concerns a VUS in genes related to the fetal phenotype. The benefit/risk ratio should be carefully assessed, since a VUS identified in an indication-relevant gene might impact current and/or future pregnancies (Mellis et al., 2018), but uncertainties may cause parental anxiety without immediate benefit for decision-making (Ferretti et al., 2019). ACMG recommend reporting VUSs related to autosomal recessive diseases when they are congruent with phenotype and a P/LP variant has been identified in the other allele (in "trans" with a pathogenic/likely pathogenic variant) (Monaghan et al., 2020). In our recent performed meta-analysis, the pES VUS rate was 8.32% in Group A, whereas it ranged between 1.44% and 10.47% in Group B (Mastromoro et al., 2022). Consultants present different reactions after receiving information of unclear significance, which varies according to one's own experiences, perceptions and family planning (Richardson et al., 2018) and sometimes they feel reassured when inherited variants are found (Lou et al., 2020). Parental analysis is crucial for the interpretation of the results and for formulating recurrence risks. VUSs and IF can present a clinical impact on both fetus and parents and VUS carriers should be evaluated after birth (Klapwijk et al., 2021) or if the couple is planning new pregnancies (Mellis et al., 2018), even if no further phenotypic findings emerge. If the alteration is parentally inherited, instrumental analyses may be proposed for the carrier. To complicate things, genetic findings are always reported under the mother's name, consequently, it can be impracticable to retrieve data during postnatal examinations (Guadagnolo et al., 2021).

2. AIM

The main purpose of this PhD project is to study prenatal CNS anomalies and the associated genetic diseases through the application of a trio-based prenatal exome sequencing (pES) approach, calculating the incremental diagnostic yield after inconclusive result of karyotype and CMA analysis, to improve their diagnosis and prognosis and to support genetic counselling. Firstly, we performed a prospective study on application of trio-pES between 1-1-2019 and 31-12-2022 on a cohort of fetuses with apparently isolated or not insolated CNS anomalies. Secondly, we performed a systematic review and meta-analysis on the published cohorts of fetuses specifically selected for CNS anomalies, with the aim to evaluate the pooled diagnostic incremental yield over karyotype and CMA, in fetuses affected by CNS anomalies and undergoing prenatal genome wide sequencing analysis in literature.

3. MATERIALS AND METHODS

3.1 Cohort 2019-2022

3.1.1 Subjects selection

Between January 1st 2019 and December 31st 2022, a prospective cohort of fetuses presenting CNS anomalies was enrolled at Policlinico Umberto I Hospital, Sapienza University of Rome. A total of 290 invasive procedures were performed for fetal US anomalies (malformations or multiple soft markers) in the II level Prenatal Diagnosis Centre of Policlinico Umberto I Hospital: 31 villous sampling and 259 amniocenteses. Among these, 5 fetuses presented CNS anomalies in the 1st trimester US scan and underwent Villous Sampling and 46 fetuses presented CNS anomalies in the II/III trimester US scan and underwent amniocentesis.

A total of 51 fetuses (from 50 pregnancies) presenting with CNS anomalies were referred to genetic counselling. Fetuses presenting CNS anomalies at 1st trimester without indication to perform pES (*i.e.* acrania, anencephaly), fetuses presenting only 1 soft marker (*i.e.* choroid plexus cysts), fetuses without subsequent confirmed diagnosis at US or fetal cerebral MRI, and women refusing invasive procedures were excluded. Pregnant women with fetal apparently isolated or non-isolated CNS anomalies identified and/or confirmed at I/II/III trimester screening US examinations in the II level Prenatal Diagnosis Centre of Policlinico Umberto I Hospital who opted and consented for invasive testing (amniocentesis or villous sampling) were included. A total of 33 fetuses were enrolled.

3.1.2 Imaging Characterization

CNS anomalies were identified at screening US examinations. A second line US examination (neurosonography) was performed in the II level Prenatal Diagnosis Centre of Policlinico Umberto I Hospital to confirm the diagnosis and to investigate other possible associated fetal anomalies. All US evaluations were performed with a Voluson 730 Expert GE or Samsung Elite WS80A machine. The anomalies were defined as *apparently isolated* CNS anomalies, in the absence of other malformations or in the presence of not more than only one minor anomaly, *non-isolated* in the presence of at least one additional major malformation (cerebral or extracerebral), and *CNS-only related anomalies* (in presence of one or more CNS anomalies).

In CNS anomalies presenting at II trimester, at least one Fetal cerebral MRI was performed in all cases in the II trimester, and, when required, a second Fetal cerebral MRI was performed in the III

trimester. MRI examinations were acquired using a 1.5 T Magnet (Siemens Magnetom Avanto, Erlangen Germany) without maternal–fetal sedation with one or two surface coil phased arrays.
The study protocol included the following sequences with the multiplanar acquisition (axial, coronal, sagittal):

- T2-weighted HASTE: repetition time (TR) 1500 ms, echo time (TE) 151 ms; slices of 3 mm; FOV 260 × 350 mm; 256 × 256 matrices; time of acquisition (TA) 20 s.
- T1-weighted FLASH 2D: TR 362 ms; TE 4.8 ms; slices 5.5 mm; flip angle 70°; FOV 350 × 300 mm; 256 × 192 matrices; TA 25 to 30 s with and without fat saturation.
- Diffusion weighted imaging: TR 8000 ms; TE 90 ms; inversion time 185 ms; slices of 5 mm; FOV 420 × 300 mm; 192 × 192 matrix; TA 45 s; 3 b-factor per floor: 0.200 and 700 mm^2/s.

The following parameters were evaluated: biometry (Fronto-Occipital Diameter, cerebral biparietal diameter, Transverse Cerebellar Diameter, height of the vermis, Antero-Posterior Diameter of the vermis and length of CC, ventriculomegaly, gyration, and signal intensity.

3.1.3 Invasive Procedure and Genetic Counselling

The pregnant women were referred for consecutive consultations in the II level center for prenatal diagnosis of Policlinico Umberto I Hospital: firstly with a fetal medicine specialist and with a clinical geneticist with expertise in prenatal diagnosis and, when required, with a pediatric neurologist also.
Informed consents for DNA storage and genetic analyses were obtained for each fetus and his parents; permission to publish imaging data was given for all subjects reported in this work and all the followed procedures were in line with the Helsinki declaration's principles of 1975, as revised in 2000. Amniocentesis and Villous Sampling were performed in Prenatal Diagnosis Centre of Policlinico Umberto I Hospital.
Parental blood samples were collected for DNA extraction and fetal DNA was obtained from amniotic fluid and chorionic villi cultures and direct extraction. In all cases fetal standard karyotyping was performed on cultured cells. In cases of chorionic villi analysis, both short-term and long-term cultures were performed. In all cases CMA was performed from DNA directly extracted from uncultured samples. Trio-pES was offered as part of the diagnostic management, in karyotype and CMA inconclusive cases after specific and extensive informed consent, genetic counselling and clinical evaluation.

3.1.4 Cytogenetic Analysis

Standard Karyotype Analysis on amniocytes was performed at Cytogenetics Laboratory at Policlinico Umberto I Hospital. Amniocytes were cultured in Chang C medium supplemented with L-glutamine (200 mM) and 1% penicillin-streptomycin, at 37 °C with 5% CO2. All karyotyping were carried out using a G-banding technique, on 20 metaphases from 2 independent cultures, according to standard guidelines.

Standard Karyotype Analysis on chorionic villi was performed at Mendel Laboratory-Cytogenetics Unit, Casa Sollievo della Sofferenza Foundation, through both short-term and long-term cultures. In short term culture, a direct preparation of uncultured, spontaneously dividing cytotrophoblast cells following a limited period of incubation were analyzed. Cell of the cytotrophoblast, were synchronized and arrested in mitosis after a short incubation period, and metaphase spreads were prepared and analyzed. In the culture method (long-term cultures), to prepare a suspension of mesenchymal cells for culture, villi were disaggregated by mechanical and enzymatic methods, and the resulting cell suspension was used to establish primary cultures. The trophoblastic layer was firstly removed by treatment with trypsin/EDTA. The villi were evaluated under an inverted microscope to check for dissociation of the trophoblast layer. Then the intercellular matrix of the core was digested by treatment with collagenase. Mesenchymal cells attach were cultured for 7-14 days, and then treated with colcemid to induce mitotic arrest. Following swelling in a hypotonic sodium citrate solution, the cells were fixed and processed for staining and analysis, manually. Chromosomes were stained using the trypsin-Giemsa banding procedure and for each case, a total of twenty cells were counted and five cells were analyzed, using cells from both primary cultures.

3.1.5 Microarray Analysis

Genomic testing for CNVs was performed at Mendel Laboratory-Cytogenetics Unit, Casa Sollievo della Sofferenza Foundation. Fetal DNA was directly extracted from amniocytes and in one case from chorionic villi, and parental DNA was extracted from blood circulating leukocytes. Microarray analysis was performed using a genomic oligonucleotide-array (180K; Agilent Technologies Inc., Waldbronn, Germany) according to standard protocol with some modifications. In brief, fetal and reference DNA were heat-fragmented and labelled with Cy5-dCTP and Cy3-dCTP (Agilent Technologies Inc.), respectively. After washes, dried slides were immediately scanned on the Agilent scanner and analysed using Cytogenomics software (version 5.1.2.1; ADM-2 algorithm; release

hg19) (Agilent Technologies Inc.). Called CNVs were represented by at least three consecutive probes, with an effective resolution of 75 Kb, and were confirmed by real-time quantitative PCR.
In one case the microarray analysis was performed using a SNP (Single Nucleotide Polymorphism) microarray platform (Cytoscan HD chip; Thermo Fisher Scientific, Waltham, MA, USA). The analysis was performed following the manufacturer's instructions. Data analysis was conducted using the Chromosome Analysis Suite software (ChAS; version 4.2.1; Thermo Fisher Scientific).
In accordance with ACMG and ClinGen guidelines, the detected CNVs were classified as pathogenic (P), likely pathogenic (LP), variants of uncertain significance (VUS), likely benign (LB) or benign (B) (Riggs et al., 2020).

3.1.6 pES: Data analysis, filtering and prioritization and variants validation

Trio-pES was offered in all karyotype and CMA inconclusive cases. In cases who accepted this further test (n=15), pES was performed always in trio (parents and fetus) through CES on genomic DNA extracted from amniotic fluid or villous sampling and parental leukocytes at Ospedale Pediatrico Bambino Gesù Medical Genetics Laboratory. Library preparation and targeted enrichment were performed using the Twist Human Core Exome Kit (Twist Bioscience, San Francisco, CA) according to the manufacture's protocol and sequenced on the Illumina NovaSeq 6000 platform. Fastq files were aligned to the human reference GRCh37/hg19. The BaseSpace pipeline (Illumina, San Diego, CA) and the TGex software (LifeMap Sciences, Alameda, CA) were used for variant calling and annotating variants, respectively. Functional impact of variants was analyzed by Combined Annotation Dependent Depletion (CADD, https://cadd.gs.washington.edu) V.1.3, Sorting Intolerant from Tolerant (SIFT, https://sift.bii.a-star.edu.sg), and Polymorphism Phenotyping v2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/). Rare variants with Minor Allele Frequency (MAF) <0.1% were filtered based on the Genome Aggregation Database (gnomAD) (http://gnomad.broadinstitute.org/), dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP), 1000 Genomes Project (http://www.internationalgenome.org/), EVS (http ://evs.gs.washington.edu/EVS/). Imaging manifestations for each fetal sample were transformed to related Human Phenotype Ontology (HPO) terms (https://hpo.jax.org/app/). Based on the ACMG guidelines (Richards et al., 2015), a minimum depth coverage of 30X was considered suitable for analysis. Variants were examined for coverage and Qscore (minimum threshold of 30), and visualized by the Integrative Genome Viewer (IGV). Sanger validation was performed on fetal DNA and parents using the Big Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI3130 GeneticAnalyzer (Applied Biosystems). In accordance with the ACMG guidelines (Richards et al., 2015) the detected

variants were classified as P (class 5), LP (class 4), VUS (Class 3), LB (Class 2) or B (Class 1). LB or B variants were not reported for the fetus and parents. VUSs were reported for the fetus and parents. To report IF and SF an opt-in or opt-out option was proposed to the couples during genetic counselling and was declared in the informed consent. In all cases, genetics results were explained during a consultation with a clinical geneticist with expertise in fetal medicine.

3.1.7 pES: variants interpretation

Variants interpretation was carried out with a multidisciplinary diagnostic process including an accurate clinical characterization, considering the evolution of the ongoing pregnancy and involving a collaboration between molecular biologists, clinical geneticists, senior sonographers, prenatal obstetricians' gynecologists, radiologists, and pediatric neurologists when required. Variants were interpreted to establish the clinical significance and relevance and if the genetic results fitted with the fetal imaging phenotypes. Genotype-phenotype correspondence was established in each case with a deep literature study, and revision of fetal cerebral MRI imaging with expertise specialists. For this study, pES diagnostic yield was scored as (n. cases with LP/P variant identified)/(eligible cases) and the VUS rate was scored as (n. cases with VUS identified)/(eligible cases).

3.1.8 Histological and Immunohistochemistry analysis in BICD2opathy

In a diagnostic case in which a diagnosis of BICD2opathy was made, histological and immunochemistry analysis were performed after pregnancy interruption. Diaphragm and spinal cord were isolated at the end of the autopsy and routinely processed for paraffin embedding. For histology, sections were stained with hematoxylin and eosin (H and E). Sections from the diaphragm were also stained with Masson's trichrome. Immunohistochemistry was performed using different primary antibodies. Sections of the diaphragm were immunostained for Myosin Heavy Chain Fast (MHCF, clone WB- MHCF, #NCL-MHCf, Leica Biosystems, Nußloch, Germany). Sections of the spinal cord were immunostained for Glial Fibrillar Acidic Protein (GFAP, clone GA5, #NCL-L-GFAP-GA5, Leica Biosystems, Nußloch, Germany), CD68 (clone 514H2, #NCL-L-CD68, Leica Biosystems, Nußloch, Germany) and NEUronal Nuclei (NeuN, clone A60, #MAB377, MilliporeTM, Darmstadt, Germany) to evaluate gliosis, to assess microglia activation and to highlight motor neurons, respectively. NeuN immunostained sections were also used to calculate the density of neurons (Number of neurons /105 μm2) in the ventral horns as described previously (Soler-Botija et al., 2002). To this end, NeuN immunostained sections were scanned via Aperio Scan Scope CS (Leica

Biosystem Imaging, Nußloch, Germany) and analyzed using the Aperio ImageScopeTM program (v12.3.2.8013). Data were compared with those previously published for terminated pregnancies of one age-matched normal fetus and one age-matched fetus affected by autosomal recessive type-I Spinal Muscular Atrophy (SMA) (Soler-Botija et al., 2002), used as a comparative model.

3.2 Systematic review and Meta-analysis

3.2.1 Systematic Review

The research was conducted following PRISMA guidelines (Page et al., 2021). We searched the Pubmed database (https://pubmed.ncbi.nlm.nih.gov/,) lastly accessed on 31 January 2023 for ("fetus" OR "fetuses" OR "foetus" OR "foetuses" OR "fetal" OR "foetal" OR "prenatal" OR "pre-natal") AND ("Central nervous system" OR "CNS" OR "brain" OR "cerebral" OR "cerebellar" OR "cerebellum" OR "vermis" OR "vermian" OR "blake" OR "Blake's" OR "hemispheres" OR "hemispheric" "hemisphere" OR "interhemispheric" OR "posterior fossa" OR "cisterna magna" OR "MCM" OR "Dandy-Walker" OR "Dandy Walker" OR "DWM" OR "hydrocephaly" OR "hydrocephalus" OR "ventriculomegaly" OR "corpus callosum" OR "callosal" OR "ACC" OR "pACC" OR "DCC" OR "Probst" OR "septo-optic dysplasia" OR "SOD" OR "cavum" or "CSP" OR "chiari" OR "acrania" OR "spina bifida" OR "anencephaly" OR "anencephalia" OR " anencephalic" OR "hydranencephaly" OR "hydranencephalia" OR "schizencephaly" OR "schizencephalic" OR "porencephaly" OR "porencephalic" OR "cephalocele" OR "encephalocele" OR "meningocele" OR "meningoencephalocele" OR "neural tube" OR "cerebrospinal fluid" OR "spinal fluid" OR "CSF" OR "NTD" OR "microcephaly" OR "megalencephaly" OR "hemimegalencephaly" OR "holoprosencephaly" OR "HPE" OR "cortical" or "cortex" OR "sulcus" OR "sulci" OR "fissure" OR "fissures" OR "gyrus" OR "gyri" OR "gyra" OR "subcortical" OR "lissencephaly" OR "cobblestone" OR "pachygiria" OR "polymicrogyria" OR "agyria" OR "heterotopia" OR "telencephalon" OR "telencephalic" OR "prosencephalon" OR "prosencephalic" OR "diencephalon" OR "diencephalic" OR "brainstem" or "brain stem" OR "mesencephalon" OR "mesencephalic" OR "pons" OR "pontine" OR "pontocerebellar" OR "medulla" OR "medullar" OR "arachnoid" OR "dural" OR "neuronal migration" OR "migrational" OR "encephalomalacia" OR "rhombencephalosynapsis" OR "grey matter" OR "white matter" OR "periventricular" OR "encephalopathy" OR "encephalopaties" OR "leukoencephalopathy" OR "acqueduct" OR "ependymal" OR "ependyma") AND ("WES" OR "CES" OR "exome sequencing" OR "Mendeliome" OR "genome sequencing" OR "GS" OR "WGS"

OR “Whole-exome” OR “Whole-genome” OR “medical-exome” OR “clinical-exome”) with a 10-year filter for publication date. A total of 486 titles and abstracts were examined. Only papers with full text available in English language were retained. Case reports and papers describing less than 5 cases were excluded. Papers not describing the application of pES or pGS based on prenatal imaging findings were excluded. Papers describing pES or pGS performed after negative targeted panels were excluded. Papers with recurrent phenotypes as an explicitly selection criterium were excluded. The number of SF and IF was collected when reported. N=10 antenatal pES and N=2 antenatal pGS cohorts (≥20–30× depth of coverage) deemed eligible for quantitative analysis. In all cases, karyotype and CMA were inconclusive.

3.2.2 Meta-Analysis

The meta-analysis was performed in collaboration with Mathematical Engineer (Politecnico di Milano University, Milan, Italy). The effect of interest was the incremental diagnostic rate of pES over karyotype and CMA. N=10 articles were deemed eligible for meta-analysis. Also, this cohort of fetuses was included for meta-analysis. We considered the diagnostic yield of pES over the number of non-diagnosed fetuses through karyotype and CMA in this cohort and in all studies included, and then pooled in a meta-analysis.

In addition, we performed a different meta-analysis considering also the two available pGS studies (≥20–30× depth of coverage) in literature for a total of N=12 articles, only including diagnostic yield related to SNVs. We underline that the SNVs included were also detectable with pES analysis. Finally, we also performed a meta-analysis considering three different subcategories when data were retrievable from studies (in the reminder cases the study was excluded from meta-analysis). The subcategories were: one isolated CNS anomaly, two or more anomalies (CNS or extra-CNS), only CNS-related anomalies (one or more).

We fitted a logistic random mixed-effects model with intercept only. The choice of a mixed-effect model, with a random component, was driven by the need of accounting for between-study heterogeneity. 95%-Clopper-Pearson confidence intervals (C.I.) for individual studies were calculated. The between-study variance (Tau-squared) was estimated through maximum likelihood estimator. In the second model, between-study heterogeneity needed further investigation. A leave-one-out analysis was performed and a Baujat plot (Baujat et al., 2002) was included. All statistical analysis were performed in R 4.2.2 (R Core Team (2022) using the meta package v6.1-0 (Balduzzi et al., 2019; Harrer et al., 2019).

4. RESULTS:

4.1 Cohort 2019-2022

We prospectively included 33 fetuses with CNS anomalies enrolled from 1st January 2019 to 31st December 2022. The median gestational age at inclusion was 20+6 GW (range 12-24 GW).

N=7 fetuses presented an apparently isolated anomaly at time of inclusion, n=16 a non-isolated anomaly (CNS or extra-CNS) and n=10 CNS-only related anomalies.

In one fetus genetic analyses were performed on DNA extracted from chorionic villi, in the remainder cases on DNA extracted from amniotic fluid. Parental DNA was extracted from peripheral blood leukocytes.

4.1.1 Cytogenetic Analysis

In 4/33 cases (12.1%) standard karyotype was conclusive and no further analysis were performed.

As expected, all four fetuses presented non-isolated anomalies, presenting with multiple malformations, cerebral and extra-cerebral (Table 2).

As expected, median gestational age at inclusion was lower in fetuses presenting multisystem malformations (19+6 GW).

In three cases, a homogeneous trisomy 13 was detected and in one case a homogeneous triploidy (Fig.4).

Phenotype	GW	Karyotype
Holoprosencephaly, hypoplastic cerebellar vermis, anophthalmia and microphthalmia, cardiopathy, polydactyly, small for gestational age	22	47, XY +13
Cerebellar hypoplasia, hypoplastic left heart, microphthalmia, micrognathia	17+4	47, XY +13
Cerebellar vermis agenesis, cerebellar lobe diastasis, small for gestational age, complex cardiopathy, cleft lip and palate	18+3	47, XY +13
Cerebellar vermis hypoplasia, micrognathia, renal bilateral hypoplasia, clubfoot, Small for gestational age, gallbladder not visualized, right aortic arch	21+3	69, XXX

Table 2: Results of Standard Karyotype.

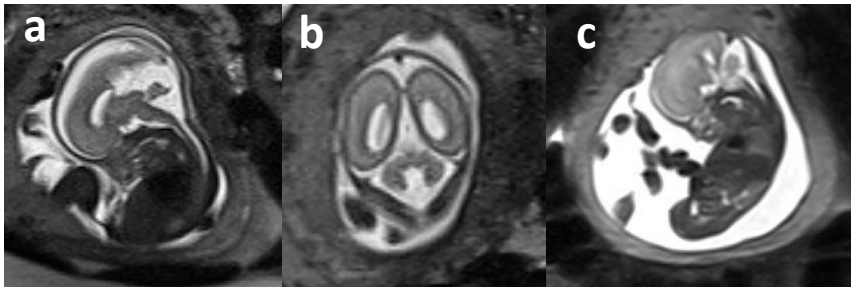

Fig.4: MRI Brain findings at 21+3 GW. In the sagittal plane (a) and coronal plane (b) is visible cerebellar lobes diastasis, cerebellar vermis uprotation with biometry at 5° percentile and (c) discrepancy between brain and fetal body.

4.1.2 Chromosomal Microarray Analysis

In 29 cases CMA was performed. In 28 cases through array-CGH and in one case through SNParray. In any case the analysis was conclusive. In two cases a CNV of uncertain significance was detected. In the first case, it was a 22q13.31 microduplication of paternal origin, in a male fetus presenting with borderline ventriculomegaly and Blake's pouch cyst at 23+2 GW (Table 3).

In the second case, it was a 12q23.3 microtriplication of maternal origin in a male fetus presenting CC hypoplasia (Table 3).

In a third case, a 16p11.2 microdeletion of paternal origin was detected, in a male fetus presenting with bilateral borderline ventriculomegaly, cardiac hypertrophy and pericardial effusion at 20+3 GW (Table 3). This microdeletion is a well-known CNV described in literature in association to neurodevelopmental phenotypes with reduced penetrance, not interpreted as explicative of the fetal phenotype.

Phenotype	GW	Karyo	CMA
Bilateral ventriculomegaly, cardiac hypertrophy and pericardial effusion	20+3	46,XY	16p11.2x1 (29673954-30197341)x1pat
Ventriculomegaly, Blake pouch cyst	23+2	46,XY	22q13.31 (47404026-48090005)x2pat
Hypoplastic Corpus Callosum	21+5	46,XY	12q23.3(104733524-105158367)x3mat

Table 3: CMA inconclusive results

4.1.3 pES Diagnostic Yield and VUS rate

In 29 cases with karyotype and CMA inconclusive results, trio pES was offered. In 15 cases couples accepted this further analysis. 14 couples declined to perform this analysis due to 1) the legal limit in Italy regarding their decision for continuation or termination of the pregnancy and/or 2) the uncertainty about the possibility to detect VUSs in genes linked to the fetal phenotype and the challenging interpretation of them. In the remainder 15 cases pES was successfully achieved.

The median delay for pES results was 13,4 days (range 11-22 days). The genotype-phenotype possible correlation was interpreted by a multidisciplinary clinical approach consisting of geneticists, senior sonographers, MRI radiologists, and obstetrician gynecologists to establish the clinical significance and relevance of the fetal imaging phenotypes.

In 5/15 cases, pES analysis disclosed conclusive results, with the detection of at least one LP or P variant interpreted as explicative of the fetal phenotype. Thus, pES provided additional genetic diagnosis in 33% of cases compared with fetal karyotyping and CMA alone, with an incremental diagnostic yield of 33.3% in this cohort.

In 10/15 (66.7%) pES analysis resulted in inconclusive results: in 6/15 (40%) cases multiple VUSs in single fetuses were detected, in 3/15 cases (20%) any variant was detected and in 1/15 (6.7%) cases, both a LP variant and VUSs variants were detected, interpreted as only partially explicative of the fetal phenotype.

The pregnancy outcome was in 10 cases termination of pregnancy, in 3 cases live birth, and follow-up was not available in 2 cases.

In 3 cases (3/13= 23%), pES results influenced the couple decision-making process during pregnancy, in one case of nondiagnostic result to continue the pregnancy, in one case of diagnostic result to terminate the pregnancy and in one case of VUSs detection and any pathogenic variant detection, to continue the pregnancy.

In all 5 case, pES result allowed to give an appropriate risk recurrence for futures pregnancies of the couples, ranging from low risk for the cases in which a *de novo* variant was disclosed, to 25% for autosomal recessive conditions and 50% for autosomal dominant condition.

This information was also useful to orient future reproductive couples' choices, with the possibility to opt for medically assisted procreation techniques and pre-implantation diagnosis or early prenatal diagnosis.

4.1.4 pES diagnostic cases

In 5/15 (33%) pES analysis disclosed conclusive diagnostic results, with the detection of at least one LP or P variant interpreted as explicative of the fetal phenotype (Table 4). The single diagnostic cases (ID 1, ID 2, ID 3, ID 4, ID 5) are described in detail in the following part.

ID	Phenotype	GW	CMA	trio-pES	Interpretation
1	Meningoencephalocele, renal cysts, myocardia hypertrophy polydactyly	21+3	neg	Hom *RPGRIP1L* c.394 A>T, p.Arg132Ter parents both heterozygous	MKS 5 (OMIM #611561)
2	Progressive ventriculomegaly, thinner CC	22+3	neg	Het *NFIA* c.82_83 dup, p.Trp30HisfsTer28 father heterozygous	BRMUTD (OMIM #613735)
3	Hypoplastic and Dysplastic Corpus Callosum, Dysmorphism of Frontal Horns and widening of interhemispheric space	21	neg	Het *ARID1A* c.2058 A>T, p.Arg1020Trp *de novo*	CSS2 (OMIM #614607)
4	Arthrogryposis, prefrontal edema and bilateral pyelectasia	22+3	neg	Het *BICD2* c.1636_1638delAAT; p. Asn546del *de novo*	SMALED2B (OMIM #628291)
5	Semi lobar holoprosencephaly	12+6	Neg.	Het *ZIC2* c.202G>T;p.Glu68Ter *de novo*	HPE5 (MIM #609637)

Table 4: trio-pES diagnostic results

ID 1: a 29 year-old primigravida was referred at 21+3 GW after US detection of meningoencephalocele, renal cysts, myocardial hypertrophy and polydactyly. The healthy couple referred non-consanguinity. Karyotype resulted 46,XX and CMA resulted negative. Trio-pES

disclosed the homozygous NM_015272.5 (RPGRIP1L): c.394 A>T, p.Arg132Ter variant in the fetus, classified as pathogenic (class 5) according to current ACMG guidelines (Richards et al., 2015). This variant was not present in gnomAD (v.2.1.1), it is reported in ClinVar (ID:1072) and it is reported in literature (Delous et al., 2007). Segregation analysis through Sanger Sequencing confirmed the presence of the heterozygous NM_015272.5 (RPGRIP1L): c.394 A>T, p.Arg132Ter variant in each parent, confirming the autosomal recessive diagnosis of Meckel Syndrome, Type 5 (MKS 5 OMIM 611561), consistent with the fetal phenotype. Given the US anomalies, the couple decided to terminate the pregnancy, independently from the pES result. However, this result allowed to establish a risk recurrence of 25% for each future pregnancy of the couple, useful for future reproductive choices.

ID 2: A 32 year-old primigravida, was referred at 22+3 GW, after US detection of progressive triventricular hydrocephalus and thinner CC. The couple was non-consanguineous.
MRI at 22 GW confirmed the US diagnosis. Karyotype resulted 46,XY and CMA resulted negative. Trio-pES disclosed the heterozygous NM_001134673 (NFIA):c.82_83 dup,p.Trp30HisfsTer28, classified as likely pathogenic (class 4) according to current ACMG guidelines (Richards et al., 2015) and inherited from the father, affected by congenital hydrocephalus, mild developmental delay and cACC. This variant was absent in gnomAD (v.2.1.1) and not previously described in literature.
NFIA pathogenic variants cause Brain Malformations With Or Without Urinary Tract Defects (BRMUTD OMIM 613735), inherited in an autosomal dominant fashion.
The molecular diagnosis was consistent with the fetal and parental (father) phenotype. Subsequent fetal cerebral MRI at 25+3 GW, also disclosed frontal polymicrogyria in the fetus. Given the imaging findings and the impossibility to predict the postnatal neurodevelopmental phenotype, the couple decided to terminate the pregnancy abroad, independently from pES result. However, this result allowed to establish a risk recurrence of 50% for each future pregnancy of the couple, useful for future reproductive choices.

ID 3: a 31 year-old pregnant woman, was referred at 21 GW after US detection of hypoplastic and dysplastic CC, dysmorphism of frontal horns and widening of interhemispheric space.
The healthy non consanguineous couple reported a previous spontaneous termination of pregnancy in the first trimester. MRI confirmed the US findings. Karyotype resulted 46, XX and CMA resulted negative. Trio-pES analysis disclosed a *de novo* heterozygous NM_006015 (ARID1A): c.3058 A>T, p.Arg1020Trp, classified as likely pathogenic (class 4) according to current ACMG guidelines

(Richards et al., 2015). The variant was nor reported in literature and absent from the gnomAD (v.2.1.1) database.

Pathogenic variants in *ARID1A* are associated with Coffin-Siris Syndrome 2 (CSS2 OMIM 614607) inherited in autosomal dominant manner. Given the fetal phenotype and the disclosed variant, the molecular diagnosis was interpreted as consistent with the US findings. The couple decided to terminate the pregnancy. pES result influenced decision-making during the pregnancy and consented to establish a low risk of recurrence (~ 1%) for future pregnancies of the couple.

ID 4: A 33-year-old primigravida was referred for a US suspicion of AMC. US at 22 GW showed a male fetus with fixed extended lower limbs and flexed upper limbs, bilateral clubfoot and bilateral semiclenched hands. Fetal movements were absent. Biometric measurements were in the normal range. Prefrontal edema was noted (6 mm, normal values at 22 GW 3.81 ±0.62 mm) as well as bilateral pyelectasis. Amniotic fluid was in the normal range.

Karyotype resulted 46,XY and CMA resulted negative. Trio-pES analysis detected a *de novo* heterozygous variant NM_015250.3 (BICD2):c.1636_1638delAAT,p.Asn546del, classified as pathogenic (class 5) according to the ACMG guidelines.

It was not reported in the gnomAD v2.1.1 database. The variant was reported in the HGMD as "Disease Mutation" (accession CD1814610), and in the ClinVar database (ID 422408) as "Likely pathogenic". It was predicted to have high deleteriousness (CADD score = 21.5) and it was previously reported postnatally in two patients (Koboldt et al., 2018) with a severe phenotype. Protein modeling performed on the p.Asn546del variant showed altered protein structure (Koboldt et al., 2018).

Given the fetal US phenotype the couple decided to terminate the pregnancy, independently from pES results. After pregnancy interruption at 22 GW, a babygram as well as an autopsy were performed. Babygram showed multiple joints contractures, bilateral clubfoot and clubhand with camptodactyly and bilateral genu recurvatum. Sloping acetabular roofs with superolateral displacement of the femoral heads were evident. At autopsy, external examination was consistent with AMC. Visceral examination confirmed bilateral pyelectasis in the absence of other grossly recognizable anomalies. Histological examination of the diaphragm (Fig. 5 a,b) revealed features of denervation atrophy. Masson's Trichrome stain (Fig. 5, b) revealed absence of endomysial fibrosis and enlargement of the perimysium, features usually found in type I SMA cases (Dubowitz et al., 2021). By MHCF immunostaining, the pattern was similar to type I and type II SMA pathological changes (Dubowitz et al., 2021). Indeed, most stained fibers were small, indicating they were type II fibers (insert in Fig. 5, b). Only few sparse larger fibers failed to stain for MHCF (insert in Fig. 5, b, arrows), suggesting that they expressed slow myosin, but the presence of hybrid fibers was not

assessed. The spinal cord showed a normal general architecture. Indeed, the ependymal lined central canal was patent, and the dorsal median septum was properly formed. In the ventral horns, neurons were histologically easily recognized (Fig.5, c) and highlighted by NeuN immunostaining (Fig. 5, d). Motor neurons were evaluated at multiple levels.

Although it is difficult to assess neuronal morphology on autopsy samples due to post-mortem artifacts, peripheral displacement of the nucleus and reduction of Nissl' granules, consistent with chromatolysis was observed in some of them (insert in Fig. 5, c). Inflammatory cells were absent. A normal staining pattern of white and gray matter was observed with GFAP (Fig. 5, e) and CD68 immunostains failed to detect gliosis and microglial activation.

Quantitative analysis revealed that neuronal density, estimated on sections immunostained for NeuN (Fig. 5, d), was markedly reduced compared to the neuronal density of one age-matched normal fetus and one age- matched type-I SMA fetus previously published (Soler-Botija et al., 2002).

This case was the first prenatal molecular diagnosis and neuropathological characterization of a severe form of BICD2-opathy in a fetus at 22 GW presenting with AMC (Marchionni et al., 2021) and pES result consented to establish a low risk of recurrence (~ 1%) for future pregnancies of the couple.

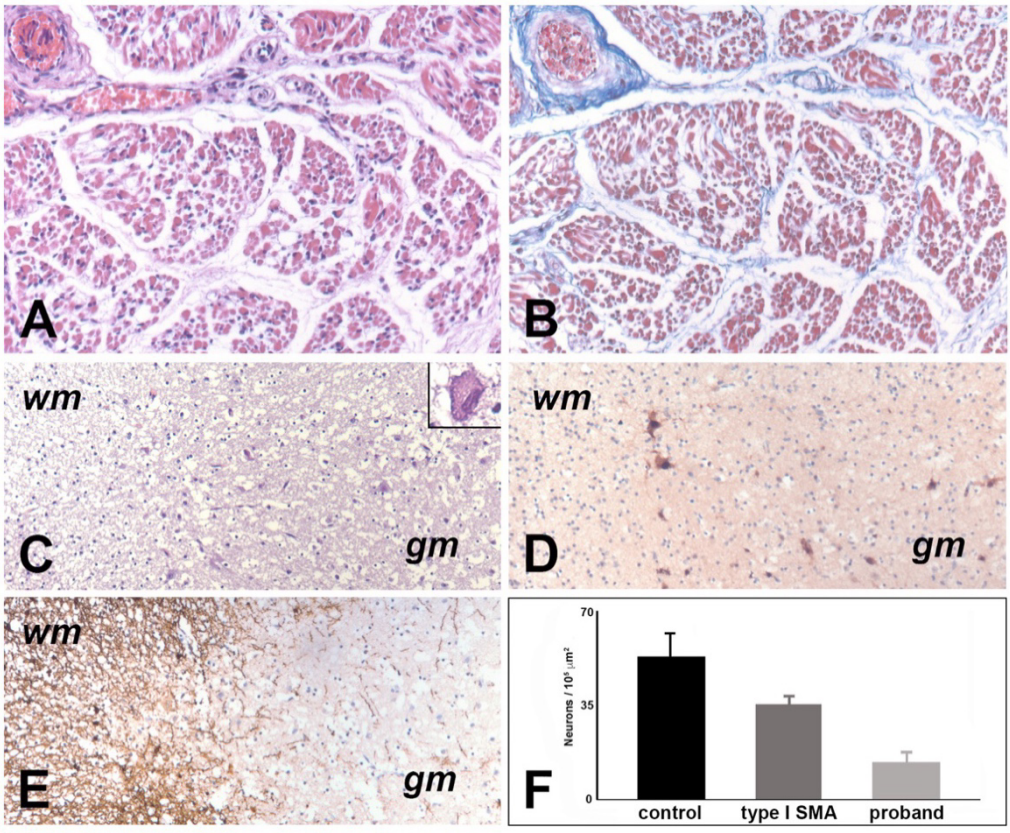

Fig. 5 Hystological and Immunochemistry results from Marchionni et al., 2021: (a) and (b) illustrate two consecutive sections of the diaphragm stained with H and E (a) and Masson's trichrome (b). Skeletal muscle fibers

are small, endomysial fibrosis is absent and perimysium is expanded. The insert in B shows the immunoreactivity for MHCF of the small atrophic muscle fibers and the negativity of the large ones (arrows). (c)–(e) illustrate seriated sections of the ventral horn of the lumbar spinal cord stained with H and E (c) and immunostained for NeuN (d) and GFAP (e) respectively. Neurons are easily recognized on H and E stained sections (c) and highlighted by NeuN immunostain (d). Some motor neurons showed peripheral displacement of the nucleus and reduction of Nissl' granules which is consistent with chromatolysis (insert in (c)). GFAP immunostaining (e) showed normal staining pattern of white (wm) and gray (gm) matter. Histogram (f) shows the density of neurons in the ventral horns of the spinal cord. Comparative data were reproduced from Soler-Botija et al. (2002). These authors generated their data through the use of anti-Hu and anti-NeuN antibodies. The obtained values were referred to be similar for the two antibodies at 22 weeks and the authors published only those obtained from Hu immunostaining. In the proband, in which the analysis was performed on NeuN immunostained sections, the density of neurons (mean ± SD, 13.72 ± 3.7) is very low compared to the density of neurons of one age- matched normal fetus (mean ± SD, 53.2 ± 8.1) and also one age-matched type-I SMA sample (mean ± SD, 35.5 ± 2.9).

ID 5: A 22 year-old primigravida was referred at 12+6 GW for US diagnosis of semi lobar HPE. Villous sampling was performed the same day and karyotype resulted 46,XY both after short-term and long-term culture. CMA performed on DNA extracted from chorionic villi resulted negative. Subsequent trio-pES analysis disclosed a *de novo* heterozygous NM_007129.5 (ZIC2):c.202G>T;p.Glu68Ter variant in the fetus, absent in both parents.

The variant was absent in gnomAD database and it was not present in literature. It was classified as LP variant according to ACMG guidelines (Richards et al., 2015), confirming the diagnosis of autosomal dominant Holoprosencephaly type 5 (HPE5; MIM 609637), consistent with the fetal phenotype. Giving the US result, the couple decided to terminate the pregnancy independently from pES result. However, pES result consented to establish a low risk of recurrence (~ 1%) for future pregnancies of the couple.

4.1.5 pES Inconclusive Cases

In 10 cases pES resulted in inconclusive findings. In 3/15 cases (20%) any variant was detected. In 6/15 (40%) cases multiple VUSs in single fetuses were detected, in 1/15 (6.7%) cases both a LP variant and VUSs variants were detected, interpreted as only partially explicative of the fetal phenotype (Table 5).

We underline that the variants classification herein reported corresponds at the time of testing, that in some cases is >1 year ago (range 2019-2022). We did not perform a new and up-to-date classification of variants as beyond the scope of this work, aimed to analyze the rate of VUSs in pES at the time of genetic counselling. The single diagnostic cases (ID 6, ID 7, ID 8, ID 9, ID 10, ID 11, ID 12) are described in detail in the following part.

ID	Phenotype	GW	CMA	trio-pES
6	Bilateral ventriculomegaly, cardiac hypertrophy and pericardial effusion	20+3	16p11.2x1 pat	het *NKX2-5* c.829G>A mat het *ELN* c.1446_1463dup pat
7	Ventriculomegaly, dysmorphic cerebellum, cerebellar vermis hypoplasia, tetralogy of Fallot, hypospadias	22+0	negative	het *SMG9* c.1547G>C pat het *CENPJ* c.2035C>A mat het *TBX20* c.456C>G mat
8	Complete agenesis of corpus callosum, suspected polymicrogyria	24+5	negative	het *IBA57* c.574G>A pat het *B3GALNT2* c.555+5G>A pat het *ARMC9* c.538_539delAT pat het *APC2* c.4520A>C mat het *HERC1* c.7615G>A mat het *ISCA1* c.248G>A mat
9	Partial agenesis of corpus callosum and frontal horns dysmorphism	21+4	negative	het *CEP290* c.226G>A mat het *COASY* c.878 G>A mat het *COASY* c.1022delC pat het *PNKP* c.1009 G>C mat het *PNKP* c.1360 C>A pat
10	Hypoplastic and dysplastic Corpus Callosum	22+1	negative	het *ANKLE2* c. 1378A>G pat het *LRP2* c.2930C>A pat het *MED23* c.3557A>G mat
11	Dysgenesis of Corpus Callosum and suspected gyration delay	20	negative	het *KATNB1* c.766 G>A pat
12	Hypoplastic and Dysplastic Corpus Callosum, ventriculomegaly, delayed gyration	19+2	negative	het *DCC* c.2456-1G>C mat het *PTCH1* c.4069A>T mat het *TCTN1* c.713-2A>G mat het *CEP120* c.1937G>A mat het *PCLO* c.14678A>G mat het *PCLO* 3755C>T pat het *NALCN* c.4975G>A mat

Table 5: VUS detected in Fetuses and Parents

In case ID 6, US scan detected bilateral ventriculomegaly, cardiac hypertrophy and pericardial effusion at 20+3 GW. CMA disclosed a 16p11.2x1 paternal inherited microdeletion. This recurrent microdeletion is a well-known CNV described in literature in association to neurodevelopmental phenotypes with reduced penetrance, such as motor speech disorder, language disorder, motor coordination difficulties, psychiatric and behavioral conditions. Most affected individuals do not have intellectual disability (defined as an IQ of <70), but many have below average cognition and learning disabilities in both verbal and nonverbal domains. Obesity is a feature of this disorder and generally

emerges in childhood as well as seizures, observed in approximately 25% of individuals with the recurrent deletion (Taylor et al., 2006 updated 2021). The prenatal finding of a 16p11.2 recurrent deletion cannot be used to reliably predict the phenotype and, in this case, was not considered as explicative of the fetal US findings, even if some CNS malformations are reported in literature. Therefore, trio-pES analysis was performed, disclosing two heterozygous VUSs inherited from parents not fitting the fetal phenotype (*ELN* MIM * 130160 and *NKX2-5* MIM *600584) (Table 5). The couple decided to continue the pregnancy and the decision was influenced by pES analysis not giving conclusive pathogenic results. The fetus is live born and follow-up is ongoing to monitor neurodevelopmental milestones.

In case ID 7, US detected ventriculomegaly, dysmorphic cerebellum, cerebellar vermis hypoplasia, Tetralogy of Fallot, and hypospadias in a fetus at 22 GW. Trio-pES analysis disclosed three heterozygous VUSs inherited from the parents, two (*SMG9* MIM *613176, *CENPJ* MIM*609279) associated to autosomal recessive conditions not fitting the fetal phenotype and one (*TBX20* MIM *606061), associated to cardiac-related phenotypes, not explaining the multisystem detected fetal anomalies (Table 5). Given the US findings, the couple decided to terminate the pregnancy, not influenced by the pES result.

In cases ID 8, ID 9, and ID 10, fetuses presented not isolated CC anomalies. In three cases multiple variants in a single fetus were detected, all inherited from parents and in some cases in compound heterozygosity (Table 5). All the variants did not match with the fetal phenotypes and were classified as VUSs according to ACMG criteria (Richards et al., 2015). In two cases, the couples decided to terminate the pregnancy, independently of pES results, and in the third case we do not know the outcome, as follow-up is missing.

In ID 11 the fetus carried a single VUS (Table 5), and the couple decided to continue the pregnancy, independently from the pES result. The fetus is live born and follow-up is ongoing to monitor neurodevelopmental milestones.

In ID 12, pES analysis detected both a LP variant and VUSs variants in a single fetus interpreted as only partially explicative of the fetal phenotype. This case was referred at 19 GW for fetal mild bilateral ventriculomegaly. The mother was carrier of a Robertsonian translocation, 45,XX,rob(13q14q). Fetal Karyotyping disclosed the same translocation in the fetus. In this case, a CMA through SNParray platform was carried out, and resulted negative, excluding the presence of uniparental isodisomy and heterodisomy.

Fetal cerebral MRI at 21 GW evidenced absent ventriculomegaly, dysmorphic frontal horns, and suspected pACC. Trio-pES disclosed the heterozygous NM_005215.3: *DCC* c.2456-1G>C, p.? variant, classified as LP according to ACMG guidelines (Richards et al., 2015) and inherited from

the apparently healthy mother. Maternal cerebral MRI did not detect anomalies or malformations and neurological examination resulted within the limits.

In addition, the fetus carried multiple VUSs inherited from both parents (Table 5): a heterozygous *PTCH1* (MIM * 601309) variant inherited from the mother; a heterozygous *TCTN1* (MIM *609863) variant inherited from the mother, a heterozygous *CEP120* (MIM *604918) variant inherited from the mother; compound heterozygous *PCLO* (MIM*60491) variants inherited from the mother and father and a heterozygous *NALCN* (MIM * 611549) variant, inherited from the mother.

The following Fetal cerebral MRI at 26+3 GW did not confirm the pACC but raised the suspicion of gyration delay.

Heterozygous pathogenic variants in *DCC* (MIM *120470) are associated with isolated mirror movements and isolated pACC o cACC, either as isolated phenomena or in combination (MIM 157600) with incomplete penetrance and good prognosis, whereas biallelic *DCC* mutations cause the severe developmental split-brain syndrome (MIM 617542). Therefore, this variant could explain only partially the fetal phenotype, possibly related to the CC isolated anomaly but could not explain the associated anomalies. In addition, the mother did not show any sign of the condition at neurological and imaging examinations and fetal CC anomaly was not confirmed at the following MRI. These elements could not exclude a role of this variant, also considering the incomplete penetrance of the condition and the fact that a fetus cannot neurologically be examined. The fetus is live born and follow-up is ongoing to monitor neurodevelopmental milestones.

In 3/15 cases (20%), ID 13, ID 14 and ID 15, class 3, 4 or 5 variants (VUS, LP and P, respectively) were not detected. ID 13 was a fetus from a twin pregnancy in which only one fetus was affected by DWM confirmed by MRI at 21+4 GW (Fig.6). The pregnant woman opted for invasive procedure through amniocentesis. Karyotype and CMA analyses were performed on both fetuses, resulting negative. Trio-pES analysis was performed only in the affected feus and any variant was disclosed. The couple opted for a selective interruption of the pregnancy (only for the fetus affected by DWM), independently from pES result.

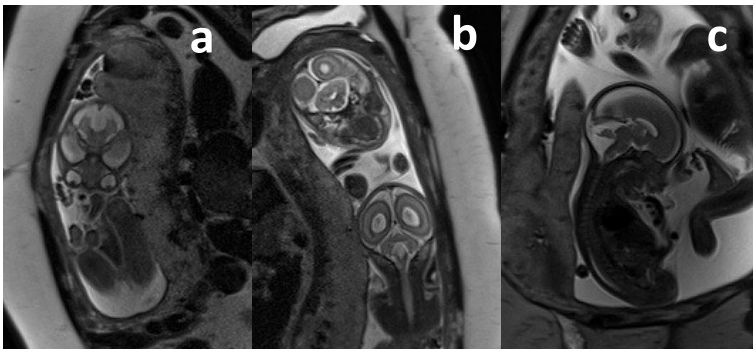

Fig. 6: MRI Imaging at 21+4 GW (twin pregnancy): DWM is visible in one of the two fetuses (a,b,c)

ID 14 was a fetus presenting cACC, pleural effusion, cardiac hypertrophy and hyperechogenic fetal bowel at 18+5 GW. Fetal MRI at 20+ 4 GW confirmed cACC and agenesis of CSP (Fig.7). Karyotype and CMA analyses were performed and resulted negative, as well as trio-pES analysis. The couple opted for termination of pregnancy, independently from pES results.

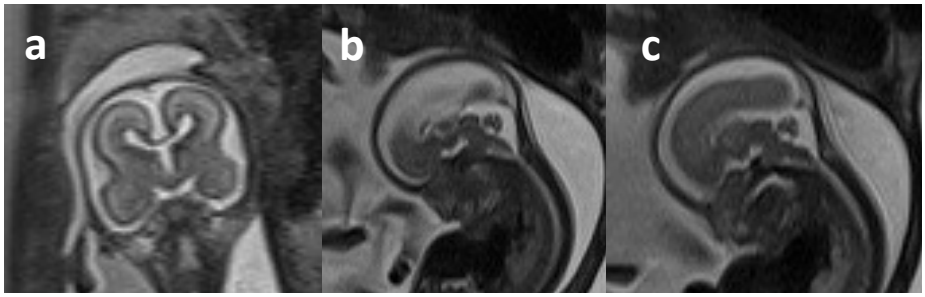

Fig. 7: MRI Imaging at 20+4 GW. Agenesis of CSP (a) and cACC (b,c)

ID 15 was a fetus presenting with hydrocephalus, CC anomaly, cerebellar hypoplasia, bilateral clubfeet, short humerus and short femur at 19+6 GW. Karyotype and CMA analyses resulted negative. This was the second pregnancy of the non-consanguineous couple with a recurrent phenotype, raising the strong suspicion of a genetic underlying condition. However, trio-pES analysis resulted negative. Further analysis in the research setting is ongoing to unravel the possible genetic cause.

4.2 Systematic Literature review and meta-analysis

4.2.1 Systematic Literature review

The review process is summarized as a PRISMA flow chart in Fig. 8.

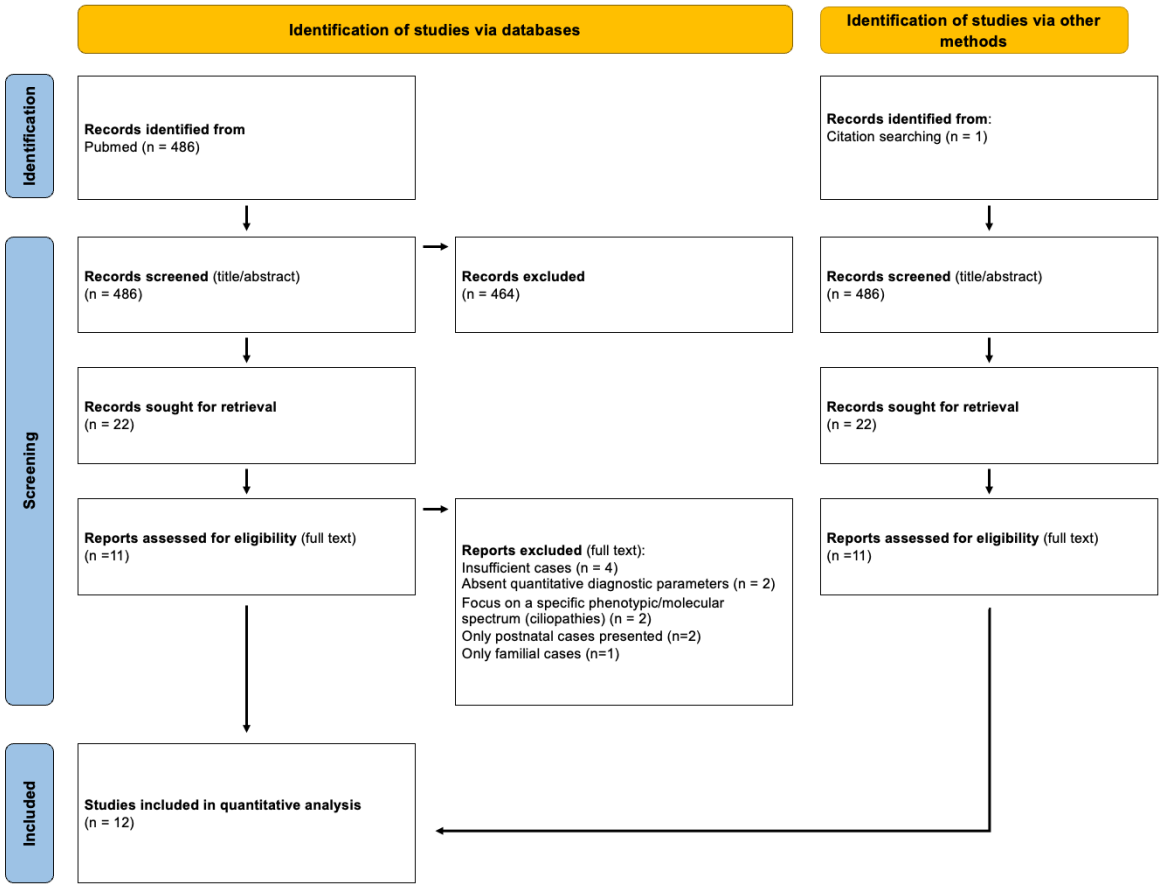

Fig. 8: PRISMA systematic review flow chart. The chart illustrates the review process performed highlighting the number of papers identified, screened, elected, and included in quantitative synthesis.

The initial search yielded 486 results. After the application of titles and abstract examination criteria from two independent reviewers, n=464 papers were excluded.

N=22 records sought for retrieval and were carefully examined: n=11 articles assessed for eligibility and n=11 were excluded. The report excluded presented insufficient cases (n=4), absent quantitative diagnostic parameters (n=2), focused on a specific phenotypic/molecular spectrum (n=2), presented only postnatal cases (n=2) or only familial cases (n=1). After examination of full text reports, through citation searching, another single article was deemed eligible, for a total of n=12 articles included in

the final review, of which n=10 on pES and n=2 on pGS (≥20–30× depth of coverage) application (Yaron et al., 2022; Lei et al., 2022; Yang et al., 2022; Liao et al., 2022; de Koning et al., 2021; She et al., 2021; Heide et al., 2020; Tan et al., 2020; Li et al., 2020; Weitensteiner et al., 2018; Reches et al., 2018; Poirier et al., 2015).

We reviewed 12 articles for a total of 428 fetuses included in the review, the characteristics of the included studies are summarized in Table 6.

We scored the diagnostic yield of pES and pGS in apparently isolated and non-isolated CNS anomalies, ranging from 18.90% to 57.14%.

We also calculated the specific representation of fetuses in four different subcategories (apparently isolated CNS anomaly, non-isolated, CNS-only related anomalies and multisystem anomalies) and the specific diagnostic yield.

The incremental diagnostic yield in the category on apparently isolated CNS anomalies ranged between 13.6% to 33.43%. The incremental diagnostic yield in non-isolated anomalies (>= 2 malformations in CNS or extra-CNS) ranged between 28.9% and 80%. The incremental diagnostic yield in the category of CNS-only related anomalies ranged between 11.9% and 60%. The incremental diagnostic yield in multisystemic anomalies ranged between 46.2 and 72.7%.

Only one paper reported IF and SF, and only five papers reported inconclusive results, explicitly declaring the number of VUSs, therefore not we did not score a specific rate due to too low number of papers reporting these findings.

Where stated we also annotated the median TAT, available in 5/12 studies (42%), which resulted 27,37 days (range 17.35-42 days) and the clinical impact of the application of pES, available in 7/12 studies (58%), which resulted influencing the decision-making process in more than half of cases (55,66%, range 26.32%-83.33%.).

We also annotated the reported termination of pregnancy (TOP) number in the papers.

Table 6: Systematic Literature Review

				DIAGNOSTIC YIELD									
Source	**Area**	**Primary Indication**	**Test**	**TOTAL**	**APPARENTLY ISOLATED** CNS anomaly +- minor anomaly	**NON ISOLATED** >= 2 malformations (cns or extra)	**CNS ONLY** one or more CNS anomalies	**MULTISYSTEMIC** Involving CNS and other systems	**TAT** (days)	**VUS**	**IF** and/or **SF**	**TOP**	**Clinical Impact**
Lei 2022	China	Corpus callosum anomalies	trio-ES	**17/50 (34.00%)**	10/34 (29.41%)	7/16 (43.75%)	n.a.	n.a	35	n.a.	n.a.	34/49 (69.39%)	17/50 (34.00 %)
Yaron 2022	Israel	CNS anomalies	quatro-WES 2 trio-WES 82 duo-WES 1 single-WES 1	**38/86 (44.18%)**	10/30 (33.33%)	28/56 (50.00%)	24/54 (46.30%)	14/32 (43.75%)	n.a.	9	n.a.	n.a.	n.a.
Yang 2022	China	CNS anomalies	WGS	**24/127 (18.90%)**	11/82 (13.41%)	13/45 (28.89%)	12/101 (11.88%)	12/26 (46.15%)	21	n.a.	3 IF 3 SF	n.a.	n.a.
Liao 2022	China	abnormal sylvian fissure	WGS	**12/24 (50.00%)**	n.a.	12/24 (50.00%)	6/15 (40.00%)	6/9 (66.67%)	n.a.	n.a.	n.a.	10/24 (41.67%)	10/12 (83.33 %)
de Koning 2021	Netherlands	CNS anomalies	trio-WES	**10/19 (52.63%)**	1/3 (33.43%)	9/16 (56.25%)	2/8 (25.00%)	8/11 (72.73%)	17.35	2	n.a.	7/19 (36.84%)	12/19 (63.16 %)
She 2021	China	Corpus callosum anomalies	trio-WES	**2/5 (40.00%)**	2/5 (40%)	n.a.	2/5 (40.00%)	n.a.	n.a.	n.a.	n.a.	5/5 (100%)	2/5 (40.00 %)
Heide 2020	France	Corpus callosum anomalies	trio-WES	**12/62 (19.35%)**	6/44 (13.64%)	6/18 (33.33%)	8/n.a.	4/n.a.	21.5	6	n.a.	9/62 (14.52%)	53/64 (82.81 %)
Tan 2020	China	CNS anomalies	trio-CES	**5/11 (45.45%)**	1/5 (20.00%)	4/6 (66.67%)	4/8 (50.00%)	1/3 (33.43%)	n.a.	n.a.	n.a.	5/11 (45.45%)	3/5 (60.00 %)
Li 2020	China	cerebellar vermis anomalies	trio-WES 11 singleton WES 8	**8/19 (41.11%)**	2/7 (28.57%)	6/12 (50.00%)	2/7 (28.57%)	6/12 (50.00%)	42	2	n.a.	10/19 (52.63%)	5/19 (26.32 %)
Weitensteiner 2018	Germany	CNS anomalies	trio-WES	**3/6 (50.00%)**	0/1 (0.00%)	3/5 (60.00%)	2/4 (50.00%)	1/2 (50.00%)	n.a.	one	n.a.	n.a.	n.a.
Reches 2018	Israel	CNS anomalies	trio-WES	**4/7 (57.14%)**	0/2 (0.00%)	4/5 (80.00%)	3/5 (60.00%)	1/2 (50.00%)	n.a.	1/7 (14.29%)	n.a.	6/7 (85.71%)	n.a.
Poirier 2015	France	Microlissence-phaly	singleton-WES	**3/12 (25.00%)**	n.a.	n.a.	3/12 (25.00%)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

4.2.2 Meta-analysis

The effect of interest of the meta-analysis was the incremental diagnostic rate of pES over karyotype and CMA. We considered the diagnostic yield of pES over the number of non-diagnosed fetuses through karyotype and CMA in this cohort and in all studies included from the systematic literature review and then pooled in a meta-analysis (Yaron et al., 2022; Lei et al., 2022; de Koning et al., 2021; She et al., 2021; Heide et al., 2020; Tan et al., 2020; Li et al., 2020; Weitensteiner et al., 2018; Reches et al., 2018; Poirier et al., 2015).

In addition, we performed a different meta-analysis considering also the two available pGS studies in literature (Liao et al., 2022; Yang et al., 2022) only including diagnostic yield for SNVs and pGS performed with ≥20–30× depth of coverage. We underline that the SNVs included are also detectable with pES analysis. Finally, we performed a meta-analysis considering three different subcategories when data were retrievable from studies (in the reminder cases the study was excluded from meta-analysis). The subcategories were: an apparently isolated CNS anomaly, non-isolated anomalies (two or more anomalies CNS or extra-CNS), and CNS-only related anomalies (one or more).

4.2.2.1 Meta-analysis of pES and pES/pGS incremental diagnostic yield

Firstly, we calculated the point estimate of the pooled incremental diagnostic yield of pES over CMA/karyotype in isolated and non-isolated CNS anomalies. The between study-heterogeneity was not too high, as suggested by the value of Thompson's I^2 =32% statistic and a not significant p-value of the Wald-Type test on Cochran's Q (p= 0.14) (Fig.9).

The pooled incremental diagnostic yield estimate was 37% (95% C.I.: [30%;46%]), as illustrated in the forest plot (Fig.9). From the fact that the point estimate is 37% and the C.I. lower bound is 30%, we conclude that the pES meta-analysis shows a substantial gain with respect to performing CMA/karyotype alone in CNS fetal anomalies (Fig.9).

Secondly, we calculated the point estimate of the pooled incremental diagnostic yield of prenatal genome-wide sequencing (from studies performing pES or including only diagnostic SNVs in studies performing pGS) over CMA/karyotype in CNS anomalies. The pooled estimate of the effect was 36% (95% C.I.: [28%;44%]), as illustrated in the forest plot (Fig.10). From the fact that the point estimate is 36% and the C.I. lower bound is 28%, we conclude that the meta-analysis including also pGS shows a substantial gain with respect to performing CMA/karyotype alone. However, a high between-study heterogeneity was detected either by a high value of Thompson's I^2 = 61% statistic and a significative p-value of the Wald-Type test on Cochran's Q ($p < 0.01$) (Fig.10).

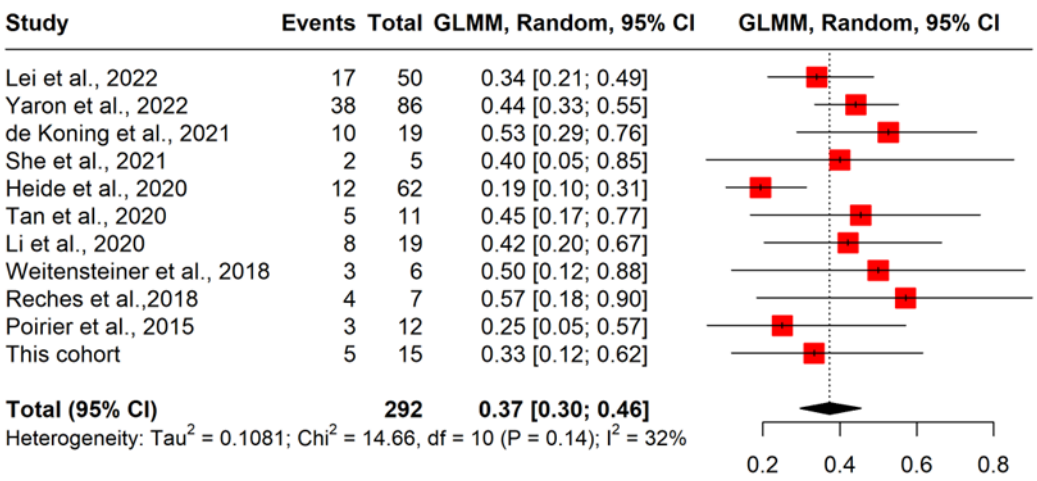

Fig. 9: Forest plot including pES studies

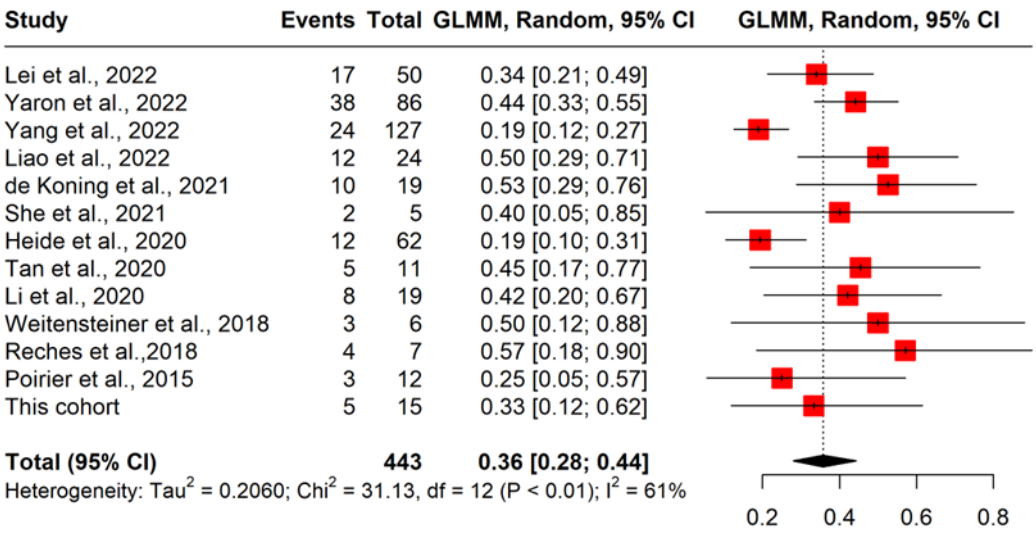

Fig. 10: Forest plot including pES and pGS studies

In general, high between-study heterogeneity could represent a limit of meta-analyses, making point estimate of the effect less reliable. Despite this, we believe this is not the case in our study, since all the works show a substantial incremental diagnostic yield with no work with values near extremes (in particular 0%). To better analyze this heterogeneity, an influence analysis of the different studies was performed. In particular, the Baujat plot (Baujat et al., 2002) was inspected (Fig. 11).

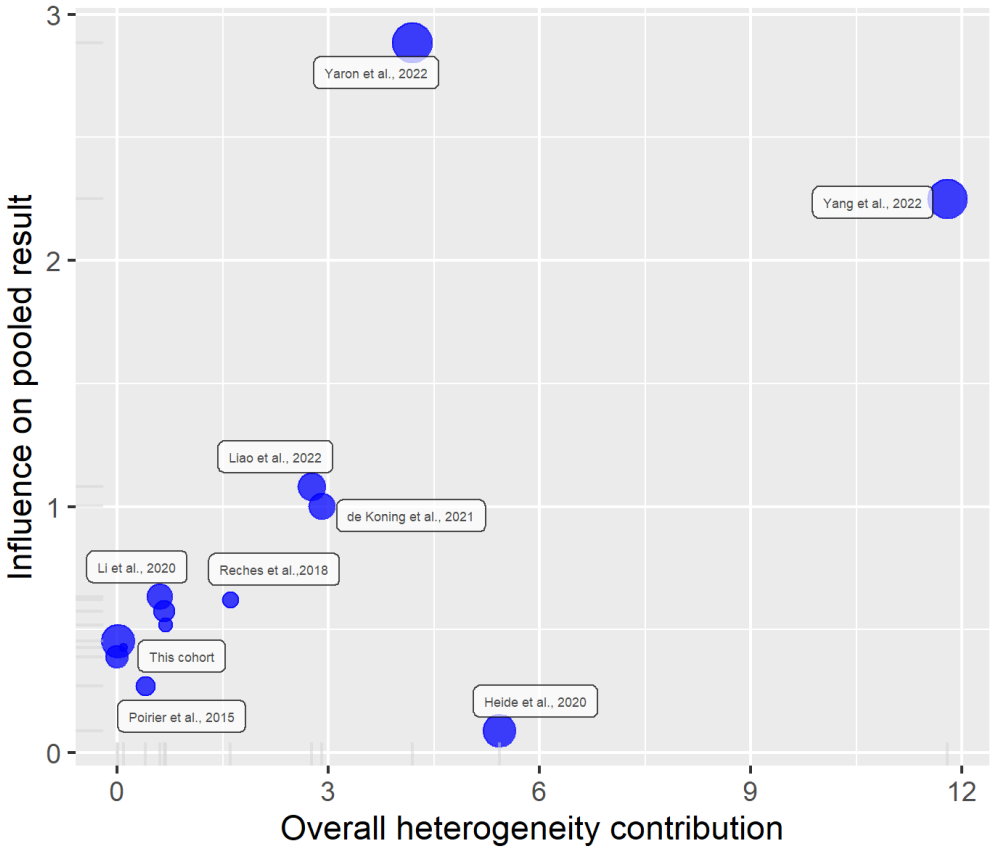

Fig. 11: Baujat plot, showing overall heterogeneity contribution

It can be immediately observed that the study by Yang et al., 2022 and Yaron et al., 2022 have a very high leveraging on the pooled result (Fig.11). Yaron et al., 2022 has a high weight on the pooled estimate, but it does not contribute substantially to heterogeneity. This is due to the fact that the corresponding cohort is large with respect to other studies, but the single-study conclusion is in line with the others. On the other hand, the study of Yang et al., 2022 represents the true outlier among all the studies. In this case, as in Yaron et al., 2022, the influence on the pooled estimate is high, reporting the largest cohort, but, conversely than Yaron et al., 2022, the contribution to between-study heterogeneity is remarkable. This can be attributed to the lower estimated diagnostic yield in the study.

To obtain a more precise insight on the leveraging of single studies that goes beyond the visual inspection of Baujat Plot (Fig. 11) a leave-one-out analysis was performed.

We fitted again the model, leaving out one study at a time. As expected, leaving out the study by Yang et al., 2022, heterogeneity drops ($I^2 = 32\%$) and the p-value of the Wald-type test on Cochran's Q is not significant ($p = 0.14$), whereas the effect does not change substantially (diagnostic yield 38%), as shown in forest plot (Fig. 12).

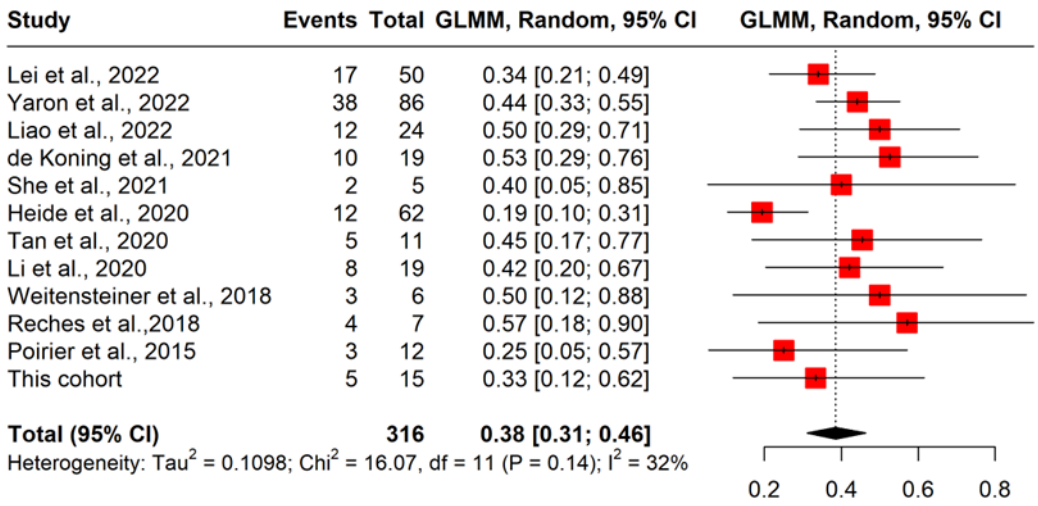

Fig.12: Forest plot leaving out the study of Yang et al., 2022

We conclude that this corroborates the validity of the result, confirming that there is a diagnostic improvement in performing genome wide sequencing analysis (either pES or pGS).

We remind that, in the case pGS is performed, our data includes only SNVs, which are also detectable through pES.

Moreover, we argue that the two studies performing pGS have not influenced our results in detecting the SNVs. Indeed, the meta-analysis considering only studies where pES was perfomed, gives comparable results for detecting SNVs with respect to the full meta-analysis not considering the outlying study by Yang et al., 2022: the point estimates are almost overlapping and the 95% C.I.s are overlapping.

Considering this result, we decided to include the two studies performing pGS in the following meta-analysis analyzing three different subcategories: apparently isolated CNS anomalies, non-isolated anomalies (two or more anomalies CNS or extra-CNS), and CNS-only related anomalies (one or more).

Notwithstanding this observation, we underline that up to date we still have a very poor representation of studies performing pGS, so further data must be collected, and our models have to be updated consequently.

4.2.2.2 Meta-analysis on subcategories of pES/pGS incremental diagnostic yield

We calculated the point estimate of the pooled incremental diagnostic yield of prenatal genome-wide sequencing (from studies performing pES or including only diagnostic SNVs in studies performing pGS) in the subcategory of fetuses presenting only an apparently isolated CNS anomaly (when it is possible to retrieve the count from the data in the papers). From data retrieved in the systematic review (Table 6), we appreciate that in this subcategory, in the two studies of Weitensteiner et al., 2018 and Reches et al., 2018, the incremental diagnostic yield is 0. However, we underline that in both studies the total number of fetuses that present a single anomaly of CNS is respectively one and two.
This fact increases substantially the uncertainty of the conclusion that there is no gain in performing pES analysis in those cases. Indeed, the number of fetuses included is too low to be representative of any gain of the diagnostic yield. We hence exclude those two studies from our sub-meta-analysis. The between study-heterogeneity was low, as suggested by the value of Thompson's I^2=19% statistic (Fig. 13). The pooled incremental diagnostic yield estimate was 22% (95% C.I.: [15%;31%]), as illustrated in the forest plot (Fig. 13). From the fact that the point estimate is 22% and the C.I. lower bound is 15%, we conclude that the meta-analysis shows a substantial gain with respect to performing CMA/karyotype alone even in presence of an apparently isolated CNS anomaly.

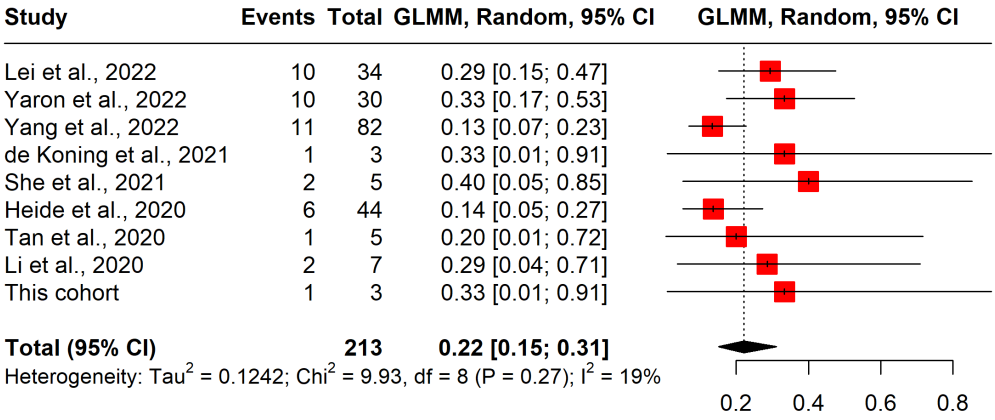

Fig. 13 Forest Plot of fetuses presenting an apparently isolated CNS anomaly

Then we calculated the point estimate of the pooled incremental diagnostic yield of prenatal genome-wide sequencing (from studies performing pES or including only diagnostic SNVs in studies performing pGS) in the subcategory of fetuses presenting non-isolated CNS anomaly (either ≥ 2 anomalies in CNS or extra-CNS) when possible to retrieve the count from the data in the papers. The

between study-heterogeneity was low, as suggested by the value of Thompson's I^2 =12% statistic (Fig. 14). The pooled incremental diagnostic yield estimate was 45% (95% C.I.: [37%;53%]), as illustrated in the forest plot (Fig. 14). From the fact that the point estimate is 45% and the C.I. lower bound is 37%, we conclude that the meta-analysis shows a substantial gain with respect to performing CMA/karyotype alone in presence of not isolated CNS anomalies, as expected.

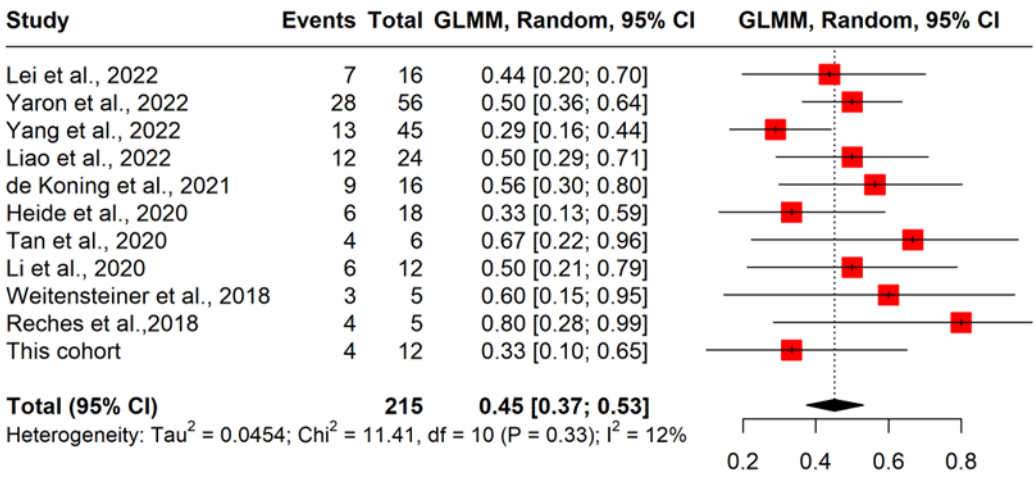

Fig.14: Forest Plot of Fetuses presenting non-isolated CNS anomalies (≥ 2 anomalies in CNS or extra-CNS)

We finally calculated the point estimate of the pooled incremental diagnostic yield of prenatal genome-wide sequencing (from studies performing pES or including only diagnostic SNVs in studies performing pGS) in the subcategory of fetuses presenting CNS-only related anomalies (one or >1) when possible to retrieve the count from the data in the papers. The between study-heterogeneity was high, as suggested by the value of Thompson's I^2 =59% statistic and a significative p-value of the Wald-Type test on Cochran's Q ($p < 0.01$) (Fig.15). However, we do not believe this can represent a limitation to the validity of the study, since we have good evidence from the complete meta-analysis that there is a gain in performing prenatal genome-wide sequencing studies in fetuses presenting CNS anomalies. The pooled incremental diagnostic yield estimate was 32% (95% C.I.: [22%;45%]), as illustrated in the forest plot (Fig. 15). From the fact that the point estimate is 32% and the C.I. lower bound is 22%, we conclude that the meta-analysis shows a substantial gain with respect to performing CMA/karyotype alone in presence of one or more than one CNS anomalies.

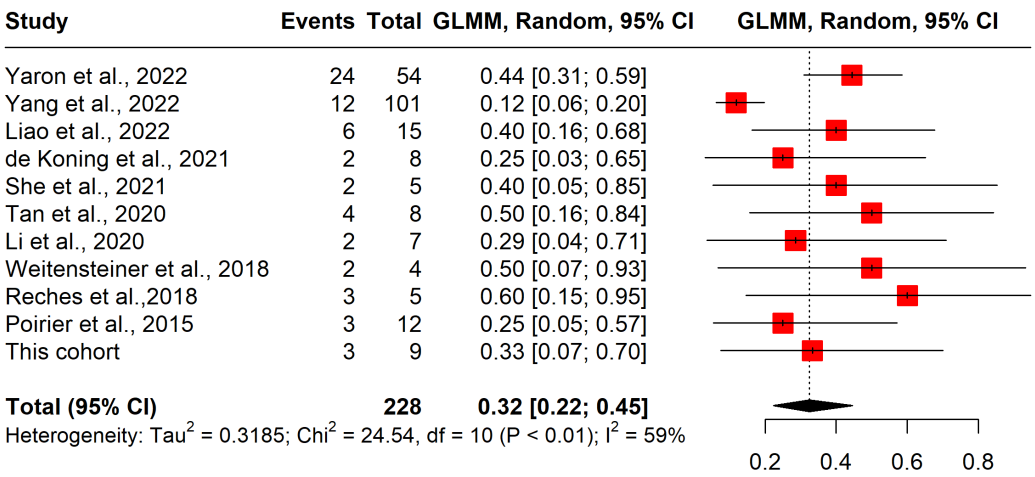

Fig.15 Forest Plot of fetuses presenting CNS-only related anomalies (one or >1)

In conclusion, meta-analysis showed a substantial diagnostic improvement in performing genome wide sequencing analyses (either pES or pGS) over karyotype and CMA alone, also in all subcategories of CNS anomalies, either apparently isolated, or associated to other CNS anomalies, or to other multisystem anomalies. The point estimate of the effect resulted 22% (95% C.I.: [15%;31%] in apparently isolated CNS anomalies category, 32% (95% C.I.: [22%;45%]) in CNS-only related anomalies (one or more) and 45% (95% C.I.: [37%;53%]) in non-isolated CNS anomalies (either ≥ 2 anomalies in CNS, or extra-CNS).

5. DISCUSSION:

5.1 State of the Art and what this study adds

NGS has revolutionized clinical practice in medical genetics, thanks to its unprecedented ability to rapidly analyze large sets of genes. Therefore, in the last years, it has had a significant impact on both genetic research and clinical diagnostics, especially in prenatal diagnosis. The first prenatal NGS-diagnosis was performed via GS on a fetus with multiple anomalies. pGS disclosed a *de novo* translocation, refining the breakpoints and disrupting *CHD7* gene, and resulting in CHARGE syndrome that would not have been established by another diagnostic method (Talkoski et al., 2012). A second case report from 2015 described how pGS was used to resolve the breakpoints of a complex chromoanasynthesis event, the prenatal GS data revealing additional information on cryptic structural events (Macera et al., 2015).

Prenatal ES was initially limited to the research field (Filges & Friedman, 2015; Hillman et al., 2015), then it has become a world-wide application analysis in selected cases, leading to a noteworthy number of case-reports and becoming a tool for the discovery of novel disease-causing genes in familial cases with lethal phenotypes (Filges & Friedman, 2015). At the beginning, only selected cases were picked up and sequenced for the high probability of monogenic disorder, resulting into a possible positive-result publication bias. The transition to the diagnostic setting was encouraged by the very high yield inferred from postnatal cases (Van den Veyver, 2016). A recent meta-analysis analysis performed by the ACMG Pediatric Exome/Genome Sequencing Evidence-Based Guideline Work Group (Manickam et al., 2021) on pediatric application of ES/GS evidenced a diagnostic yield of 38% for genome-wide sequencing, compared with the diagnostic yield of 21% for standard genetic testing. The risk ratio (RR) resulted in favor of genome-wide sequencing (RR 1.76 [95% CI 1.20–2.58]), with an even larger yield among studies that used GS (43%) than ES (34%). Therefore, in patients with congenital anomalies, developmental delay, and intellectual disability is currently recommended to consider ES/GS a second-tier test or even a first-tier test (Manickam et al., 2021).

Patients eligible for postnatal ES/GS usually display a more overt phenotype, comparably easier to dissect and investigate. The deeper phenotyping available in the post-natal setting confers stronger clinical suspicion and a solid base for molecular analysis and can grant a higher diagnostic yield. However, in the last five years, ES analysis has been increasingly adopted in the clinical prenatal setting, expanding the prenatal presentation and phenotypic spectrum of many genetic conditions.

In 2019, two large-scale sequencing projects (from cohorts selected between 2014 and 2017), were performed using trio-pES in 610 and 234 fetuses (and parental samples) with a wide range of fetal structural anomalies, after exclusion of aneuploidy and CNVs. These studies reported an overall

incremental diagnostic yield of 8.5% (Lord et al., 2018) and 10% (Petrovski et al., 2019) over karyotype and CMA. Despite being below expectations, the incremental yield of pES resulted at least comparable to that of karyotype as a first-tier test or CMA as a second-tier test.

These encouraging literature data suggested that diagnostic pES availability and its application had a pivotal role in prenatal diagnosis, though more homogeneity in access criteria was needed. In the following years, its widespread application has led to more available data, current practice is still evolving, and guidelines were not well established.

The first inter-society position statement providing a framework for pES (ISPD, SMFM, PQF, 2018) discouraged routine use of pES, and suggested its use mainly in research, with prompts for single-case evaluation. In 2020, the ACMG published a statement on pES (Monaghan et al., 2020) suggesting that, as a new diagnostic test in fetal medicine, pES might be considered when a diagnosis could not be obtained using routine prenatal methods in a fetus with one or more significant anomalies. At that time, the use genome-wide sequencing was an emerging technology, in the diagnostic work-up of pregnancies complicated by fetal congenital anomalies. This document covered a wide array of topics, from pre-test counseling to reanalysis after negative results, providing much needed prompts on debated questions like VUS, incidental and secondary findings.

However, it has not to be considered a binding guideline, being interpretable variously in each case.

The last inter-society position, published in April 2022 (Van den Veyver et al., 2022), recognized that fetal diagnostic sequencing has increased over the last five years, providing sufficient experience to allow clinical use. The statement recommends that current existing data support that prenatal sequencing is beneficial for pregnancies with a fetus having major single anomalies, for which *i.) no genetic diagnosis was found after CMA and a clinical genetic expert review considers the phenotype suggestive of a possible genetic etiology; ii.) the multiple anomaly "pattern" strongly suggests a single gene disorder with no prior genetic testing. As pES is not currently validated to detect all CNVs, CMA should be run before or in parallel with pES in this scenario* or an history of a prior undiagnosed fetus (or child) affected with a major single or multiple anomalies *with a recurrence of similar anomalies in the current pregnancy without a genetic diagnosis after karyotype or CMA for the current or prior undiagnosed pregnancy.*

The surprising indication is that this statement recommends the use of pES either after standard diagnostic testing proves uninformative or concurrently with standard diagnostic testing.

To support the suggestion to perform pES in parallel with CMA, studies focused on detection rates of pES in different fetal anomalies, interpretation of variants pathogenicity and to establish optimized indications, are needed.

A recent systematic literature review showed that pES offered for unselected pregnancies complicated by US detected fetal anomalies an overall diagnostic yield of 31% over karyotype and CMA. The diagnostic yield in unselected cohorts of fetuses with anomalies resulted 15% but increases to 42% in cases series selected because they had a higher suspicion of a single gene disorder based on the phenotypic presentation and/or family history (Mellis et al., 2022).

We have also recently performed a meta-analysis to estimate the pooled incremental diagnostic yield over CMA for P/LP variants in unselected cohorts of fetuses affected by US anomalies. The pooled incremental yield resulted 19.47% (95% C.I.[18.94–20.01%]), with 27.47% (95%C.I.[26.45–28.49%]) of diagnostic yield in the category of multiple structural anomalies (≥2) (Mastromoro et al., 2022). Although for some categories more evidence is needed to refine numbers, there are now sufficient data to start differentiating diagnostic yields by specific organ system affected.

With this aim, we decided to focus on a specific cohort of anomalies in this phD thesis, in particular CNS anomalies, to assess the incremental diagnostic yield of trio-based pES after karyotype and CMA inconclusive result. The cohort of fetuses includes apparently isolated CNS anomalies, in the absence of other not-related major malformations, fetuses presenting CNS-only related anomalies (one or more) and CNS anomalies associated with other malformations (CNS or extra CNS).

In our single-center cohort pES diagnostic yield resulted 33%, substantially increased over karyotype and CMA alone, in five cases disclosing likely pathogenic or pathogenic variants establishing a conclusive diagnosis. In the first case (ID 1), the couple was referred after US detection of meningoencephalocele, renal cysts, myocardial hypertrophy and polydactyly. Trio-pES disclosed the homozygous NM_015272.5 (RPGRIP1L): c.394 A>T, p.Arg132Ter variant in the fetus, classified as P (class 5) according to current ACMG guidelines (Richards et al., 2015). Segregation analysis confirmed the presence of the heterozygous NM_015272.5 (RPGRIP1L): c.394 A>T, p.Arg132Ter variant in each parent, confirming the autosomal recessive diagnosis of Meckel Syndrome, Type 5 (MKS 5; OMIM 611561) related to biallelic variants in *RPGRIP1L* (RPGRIP1-Like OMIM *610937), consistent with the fetal phenotype.

The second case (ID 2) was thought-provoking, since the couple was referred after US detection of progressive triventricular hydrocephalus and thinner CC. The first performed fetal MRI at 22 GW (Fig.16 a,b,c) confirmed the US diagnosis.

Trio-pES disclosed the heterozygous NM_001134673 (NFIA):c.82_83 dup,p.Trp30HisfsTer28, classified as LP (class 4) according to ACMG guidelines (Richards et al., 2015) and inherited from the father, affected by congenital hydrocephalus, mild developmental delay and ACC.

A second fetal MRI performed at 25 GW (Fig.16 d,e,f) confirmed the progressive hydrocephalus and detected polymicrogyria in the fetus. MRI in the father detected asymmetric ventriculomegaly, Chiari II malformation and ACC (Fig.16 g,h,i).

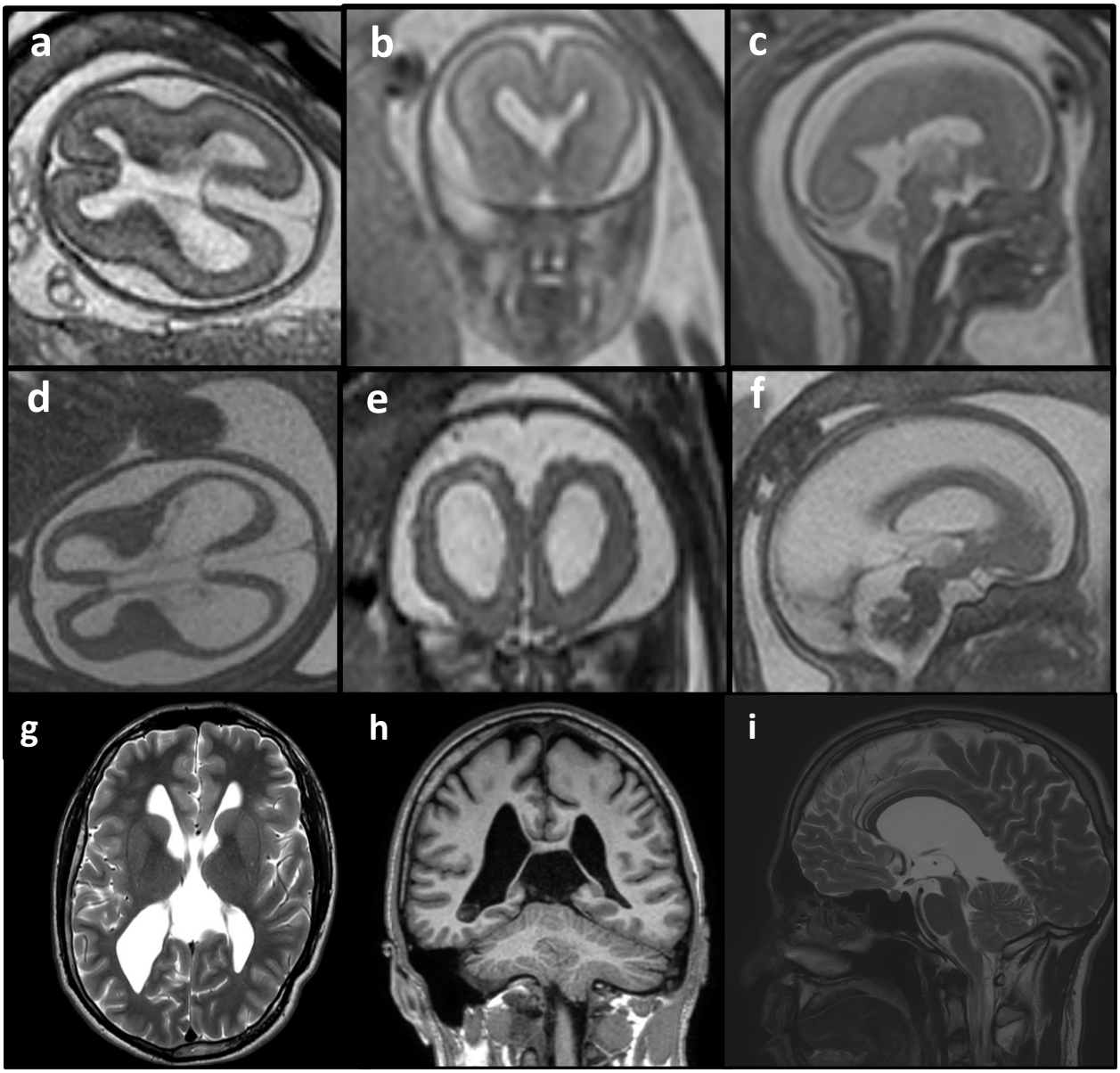

Fig.16 MRI findings in the fetus at 22 GW (a,b,c) 25 GW (d,e,f) and in the father (g,h,i).

In the fetus progressive triventricular hydrocephalus (a,b,d) and thinner CC (c,f) was detected. At 25 GW also polymicrogyria was noted (e). In the father on the axial plane is visible ventricle enlargement asymmetry (g), in the coronal FLAIR sequence, the ventricle asymmetry, CSP enlargement and Chiari II malformation (h) and in the sagittal plane ACC and Chiari II malformation (i).

NFIA (Nuclear Factor I/A OMIM *600727) pathogenic variants cause Brain Malformations With Or Without Urinary Tract Defects (BRMUTD; OMIM 613735), inherited in an autosomal dominant fashion. The molecular diagnosis was consistent with the fetal and parental (father) phenotype.

This case also illustrates how using inheritance filtering in the prenatal setting can miss fetal diagnoses. In pES often the analysis pipeline uses the assumption that both parents are unaffected and so if pipelines filter on inheritance patterns, the inherited autosomal dominant variants could be filtered out of the dataset and therefore not identified, as underlined by Chandler et al., based on their own laboratory experience (Chandler et al., 2022).

In fact, in the situation where a parent is thought to be affected with the disease, this information should be submitted to the pipeline to prevent an inheritance filter from removing any dominant variants carried by both the proband and the affected parent.

The third case (ID 3) was referred at 20 GW after US detection and MRI confirmation of hypoplastic and dysplastic CC, dysmorphism of frontal horns and widening of interhemispheric space (Fig.17). Trio-pES analysis disclosed a *de novo* heterozygous NM_006015 (ARID1A): c.3058 A>T,p.Arg1020Trp, classified as likely pathogenic (class 4) according to ACMG guidelines (Richards et al., 2015). Pathogenic variants in *ARID1A* (At-Rich Interaction Domain-Containing Protein 1A OMIM* 603024) are associated with Coffin-Siris Syndrome 2 (CSS2; OMIM 614607), inherited in autosomal dominant manner. Given the fetal phenotype and the *de novo* variant, the molecular diagnosis was consistent with the US findings.

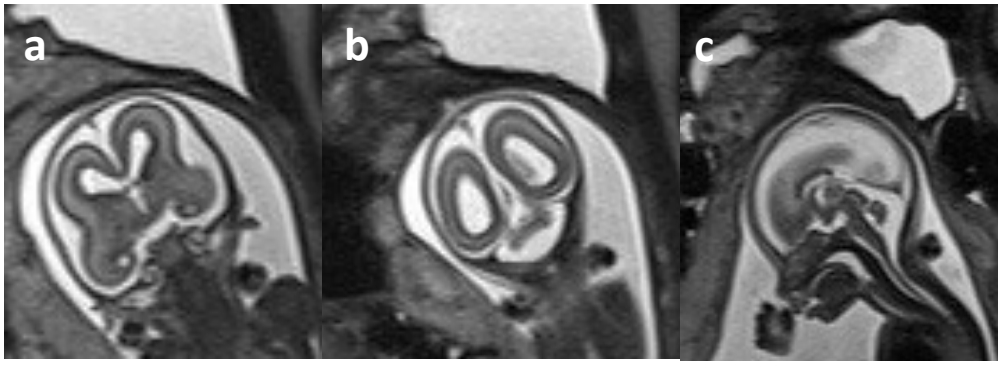

Fig.17 MRI findings at 20+2 GW: a,b,c hypoplastic and dysplastic CC, smaller CSP, dysmorphism of frontal horns and widening of interhemispheric space.

The fourth diagnostic case (ID 4) was referred for US suspicion of AMC (Fig.18 A). Trio-pES analysis detected a *de novo* heterozygous NM_015250.3 (BICD2):c.1636_1638delAAT,p.Asn546del variant, classified as P (class 5) according to the ACMG guidelines (Richards et al., 2015), previously reported in literature in a severe postnatal case (Kolbolt et al., 2018). After pregnancy interruption a babygram (Fig. 18 B and C), as well as a neuropathological study (Fig. 5) were performed,

representing the first prenatal molecular diagnosis and neuropathological characterization of a severe form of BICD2-opathy (Marchionni et al., 2021).

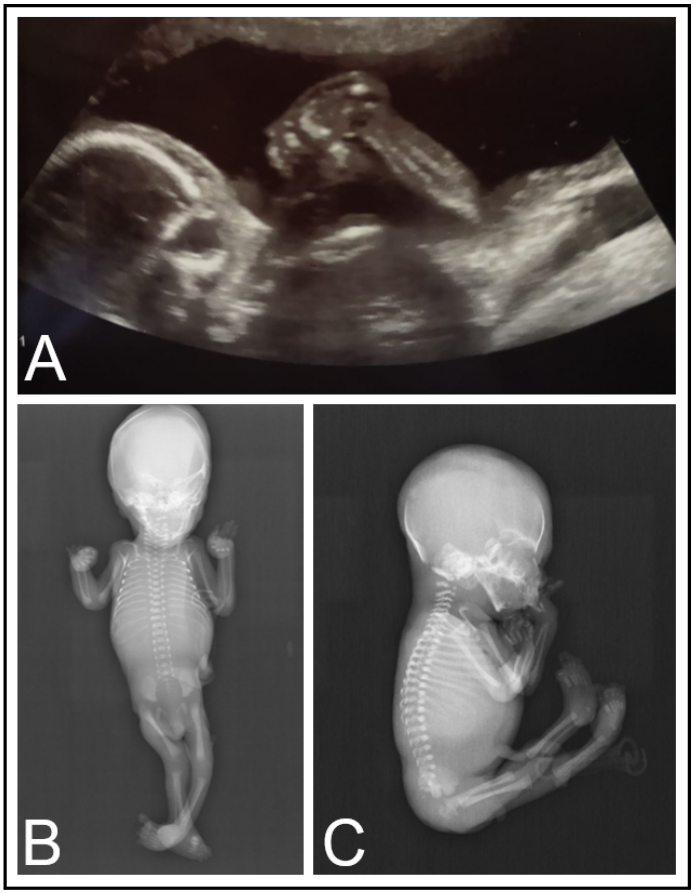

Fig. 18 Imaging findings: US at 22 GW showing prefrontal edema and fixed extended lower limbs with clubfoot (A), babygram showing multiple joints contractures, bilateral clubfoot and clubhand with camptodactyly and bilateral genu recurvatum. Sloping acetabular roofs with superolateral displacement of the femoral heads were evident (B) and (C)

AMC is characterized by the development of joint contractures in two or more areas of the body, the most severe forms being lethal in utero or after birth (Hall, 2014). Neurogenic, mechanical and myogenic-types have been described, resulting in complete or partial movement restriction and subsequent disruption of joint formation (Hall & Kiefer, 2016).

BICD2 (BICD Cargo Adaptor 2, MIM*609797) variants have been recently associated with severe prenatal-onset forms of spinal muscular atrophy, lower extremity-predominant 2B (SMALED2B MIM 618291). The majority of cases reported in literature are milder forms with childhood-onset (SMALED2A MIM 615290). In contrast to the milder form (SMALED2A) mostly transmitted from an affected parent, all but one mutation described in literature in severe cases occurred *de novo* (in

the remainder case segregation analysis was not available), confirming the association of *de novo* mutations with the most severe forms (Koboldt et al., 2020).

Despite variants in CC2 and CC3 are more often associated with severe disease than variants in CC1 (Koboldt et al., 2020), genotype–phenotype correlations are not utterly evident and other molecular mechanisms should be investigated to elucidate such a broad phenotypic spectrum.

In the fetus reported here, the histologic analysis of the diaphragm revealed features similar to type I and type II SMA, including denervation atrophy, absence of endomysial fibrosis, enlargement of the perimysium and, by MHCF immunostaining, positivity of most of the small fibers and negativity of the bigger ones (Fig. 5).

In addition, the seriousness of the phenotype at 22 GW, reflected in the very low neuron density not only in comparison to a normal age-matched fetus but also in comparison to one age-matched type-1 SMA sample (Soler-Botija et al., 2002), was consistent with a very early motor neuron disruption in SMALED2B.

These neuropathological findings supported the hypothesis that anterior horn cell loss in SMALED2 starts in early intra-uterine life and is largely complete by birth, with slowly progressive postnatal clinical course in SMALED2A and a severe outcome in SMALED2B, possibly due to the very early deleterious motor neuron disruption occurring during development.

Although more cases are necessary to confirm this hypothesis, the present case provided further insight into the clinico-pathological consequences and the extreme phenotypic heterogeneity of *BICD2* mutations in humans (Marchionni et al., 2021).

The last diagnostic case (ID 5) was the only case analyzed on a villous sampling. In fact, as previously discussed, many CNS malformations appear in the second trimester, and only in a few numbers of cases, such as HPE, diagnosis can be made earlier.

In this case, the fetus was referred in the first trimester for a US diagnosis of semilobar HPE. Trio-pES analysis disclosed the *de novo* heterozygous NM_007129.5(ZIC2):c.202G>T;p.Glu68Ter variant in the fetus, absent in both parents, confirming the diagnosis of autosomal dominant Holoprosencephaly type 5 (HPE5; MIM 609637), related to heterozygous variants in *ZIC2* (Zic Family, Member 2 OMIM *603073), and consistent with the fetal phenotype.

Despite the small cohort analyzed, these five cases represent a high incremental diagnostic yield (33%) of pES over karyotype/CMA alone. We remind that, considering epidemiological data, to obtain a cohort of 51 fetuses affected by CNS malformations (the initial cohort enrolled), it is necessary to have a catchment area of almost 25.0000 physiological pregnancies.

Precedent studies in literature, reported different diagnostic yield in the CNS subgroup retrieved from cohort studies of all kinds of unselected US anomalies. For example, Petrovski et al., retrieved from

a cohort of 243 maternal-fetal samples, 49 cases in the CNS subgroup, with a diagnostic yield of 22% (11/ 49) (Petrovski et al., 2019). In another large pES study of 610 families (PAGE study), Lord et al. reported a diagnostic yield of 4.3% in 69 fetuses with brain anomalies (Lord et al., 2019).

In our previous meta-analysis, considering, again, fetuses retrieved from unselected cohorts for all kinds of US anomalies (Group A), we retrieved data for the different subcategories (*i.e.* CNS anomalies). However, data for the meta-analysis of the specific organ anomalies were not available for all the papers included in Group A. When data were available, most papers included in the anomaly-specific subcategories both fetuses with isolated features and associated anomalies, without the possibility to discern the two categories. In addition, for some of them with more than one anomaly, the count was repeated in several subcategories with variable inclusion criteria. With these limitations, the pooled diagnostic rate resulted 24.70% (C.I. [23.94– 25.45%]) in CNS anomalies (Mastromoro et al., 2022). For Group B, including single papers describing specific anomalies, meta-analysis was not performed and, in that case, we only reviewed articles annotating diagnostic yields. Concerning CNS, in a cohort of fetuses presenting isolated anomalies, the diagnostic rate resulted 45% (Tan et al., 2020); while in another cohort of isolated and non-isolated CNS anomalies, the diagnostic rate resulted 50% (Weitensteiner et al., 2018); and in a third cohort of fetuses presenting with isolated and not isolated cerebellar vermis defects and DWM, the diagnostic rate resulted 42% (Li et al., 2020). In two cohorts of fetuses presenting CC anomalies (She et al., 2021; Heide et al., 2020), the diagnostic rate scored 40% and 19%, respectively.

Therefore, to obtain a more precise picture of the state of the art on application of pES in cohorts of fetuses selected only for CNS malformation, in this phD project, we also performed a systematic review of the literature following PRISMA guidelines (Page et al., 2021) and a meta-analysis focused on this kind of anomalies, also calculating diagnostic yield in subcategories with apparently isolated CNS anomalies, anomalies limited to CNS (one or more) or associated to other malformations.

We included 10 papers focused on the application of pES and 2 on pGS (≥20–30× depth of coverage) described in detail in Table 6 (Yaron et al., 2022; Lei et al., 2022; Yang et al., 2022; Liao et al., 2022; de Koning et al., 2021; She et al., 2021; Heide et al., 2020; Tan et al., 2020; Li et al., 2020; Weitensteiner et al., 2018; Reches et al., 2018; Poirier et al., 2015).

In this review only papers focused on CNS anomalies were included, ruling-out specific phenotypic subgroups from previously published larger cohorts (*i.e.* Lord et al., 2019 and Petrovski et al., 2019) to avoid double-counting of cases.

Therefore, after application of exclusion criteria, we reviewed 12 articles for a total of 428 fetuses included in the systematic review. We scored the diagnostic yield of prenatal genome-wide

sequencing methods (either pES or pGS) in isolated and non-isolated CNS anomalies, ranging from 18.90% to 57.14%. (Table 6).

Only one paper reported SF/IF and only five papers reported inconclusive results, explicitly declaring the number of VUSs, therefore we did not score a specific rate due to too low number of papers reporting these findings.

When stated, we also annotated the median TAT, available in 5 studies, which resulted 27,37 days (range 17.35-42 days) and the clinical impact of the application of pES, available in 7 studies, which resulted influencing the decision-making in more than half of cases (55,66%, range 26.32%-83.33%).

Then to obtain more reliable data, we pooled the results of our cohort and available literature data cohorts in a meta-analysis. The effect of interest of the meta-analysis was the incremental diagnostic rate of pES over karyotype and CMA.

Firstly, we calculated the point estimate of the pooled incremental diagnostic yield of pES over CMA/karyotype in CNS anomalies. The pooled incremental diagnostic yield estimate was 37% (95% C.I.: [30%;46%]) (Fig.9), showing a substantial gain with respect to performing CMA/karyotype alone. Secondly, as the number of studies is limited, we performed a second meta-analysis also including the two available studies in literature performing pGS and including only the detected SNVs in the count of diagnostic yield. The results were confirmed, with a pooled estimate of the effect of 36% (Fig.10) and we concluded that this corroborates the validity of the result, confirming that there is a diagnostic improvement in performing genome wide sequencing analysis (either pES or pGS) in prenatal diagnosis of CNS anomalies. We argue that the pGS analysis has not influenced our results in detecting the SNVs, as we considered only diagnosis that could have been made also by pES. Therefore, to perform following meta-analysis on subgroups, we decided to include pGS studies to have a wider fetuses representation. Notwithstanding this observation, we underline that up to date we still have a very poor representation of studies performing pGS, so further data must be collected, and our models must be updated consequently.

We also performed a leave-one-out analysis to obtain a more precise insight on the leveraging of single studies, after observing a high heterogeneity, and having inspected the Baujat plot (Baujat et al., 2002) (Fig.11). Consequently, leaving out the outlier study by Yang et al., 2022 (Fig.12), heterogeneity dropped ($I^2 = 32\%$) and the p-value of the Wald-type test on Cochran's Q became not significant ($p = 0.14$), whereas the effect did not change substantially (diagnostic yield 38%). This study performed pGS on a large cohort of unselected fetuses (n=162) affected by CNS anomalies (Yang et al., 2022). Overall, considering all the possible variants detectable through pGS, the primary diagnosis was achieved in 24 cases (diagnosis rate of 38.3%): 18 aneuploids, 21 CNVs, three small CNVs and 26 pathogenic or likely pathogenic SNVs (Yang et al., 2022).

However, as discussed, we considered only fetuses with diagnostic SNVs (n=127), with an overall diagnostic rate of 18.90% (24/127) (Table 6). We also considered the different subcategories and n=82 fetuses presented an apparently isolated CNS anomaly, n=101 fetuses presented anomalies in CNS only (one or more), while 45 had both CNS and non-CNS abnormalities. In cases of apparently isolated CNS anomalies the diagnostic rate was 13.41%, in non-isolated cases the diagnostic rate was 28.9% and in the CNS-only related anomalies diagnostic rate was 11.9%.

In the paper, authors compared diagnostic rates of aneuploidies, CNVs and SNVs and concluded that aneuploidies and single-gene variants showed the greatest contribution to primary diagnosis in their cohort, advocating that pES and deep pGS, should be considered after uninformative clinical karyotyping for these types of anomalies (Yang et al., 2022). Therefore even if this study represent the outlier of our meta-analysis, the overall conclusions are in line with our cohort result in which in 4/33 cases (12.1%) standard karyotype was conclusive, and in 5/15 cases (33%) pES disclosed a conclusive diagnosis, despite our higher diagnostic yield, probably influenced by the small size of our cohort.

We also calculated the point estimate of the pooled incremental diagnostic yield of prenatal genome-wide sequencing (from studies performing pES or including only diagnostic SNVs in studies performing pGS) in the subcategory of fetuses presenting only an apparently isolated CNS anomaly (when it is possible to retrieve the count from the data in the papers), resulting in a pooled incremental diagnostic yield estimate of 22% (95% C.I.: [15%;31%]) (Fig.13).

Then we calculated the point estimate of the pooled incremental diagnostic yield of prenatal genome-wide sequencing (from studies performing pES or including only diagnostic SNVs in studies performing pGS) in the subcategory of fetuses presenting non isolated CNS anomaly (either ≥ 2 anomalies in CNS, or extra-CNS), resulting in a pooled incremental diagnostic yield estimate of 45% (95% C.I.: [37%;53%]) (Fig.14), as expected higher than in the subcategory of apparently isolated CNS anomalies.

We finally calculated the point estimate of the pooled incremental diagnostic yield of prenatal genome-wide sequencing (from studies performing pES or including only diagnostic SNVs in studies performing pGS) in the subcategory of fetuses presenting CNS-only related anomalies (one or >1) when possible to retrieve the count from the data in the papers. The pooled incremental diagnostic yield estimate resulted 32% (95% C.I.: [22%;45%]) (Fig.15), a result in-between the incremental diagnostic yield of apparently isolated single CNS anomalies and non-isolated CNS anomalies.

In conclusion, meta-analysis showed a substantial diagnostic improvement in performing genome wide sequencing analysis (either pES or pGS) over karyotype and CMA alone, also in all subcategories of CNS anomalies, either apparently isolated, or associated to other CNS anomalies, or

in non-isolated anomalies. The point estimate of the effect resulted 22% (95% C.I.: [15%;31%] in single CNS anomalies category, 32% (95% C.I.: [22%;45%]) in CNS-only related anomalies (one or more) and 45% (95% C.I.: [37%;53%]) in non-isolated anomalies (either ≥ 2 anomalies in CNS, or extra-CNS).

Notwithstanding this observation, to correctly interpretate these results, it should be underlined that reported diagnostic yields must be tempered by several factors including the type of sequencing used, the number of genes analyzed and the laboratories' practices on defining pathogenic variants.

5.2 Benefits and Challenges

There is limited evidence on the neurodevelopmental outcomes of children affected by CNS anomalies diagnosed prenatally. Predicting an individual fetus risk of developmental difficulties is challenging. An integrated approach with neuroimaging, infectious, and genetic analyses may add useful information (Hart et al., 2022). Not all this information may be available at the time of first counselling, which should be a step-wise approach, with further discussions held as more information is obtained. With this regard, ES can add a valuable prognostic information, if a genetic cause is evidenced, adding elements in the parental decision-making process and guiding clinical management during pregnancy and the perinatal period (Dhombres et al., 2022).

For families who consider termination of pregnancy as a management option based also on knowledge from pES results, having a short result turnaround time enables them to do so within the gestational age limitations set by the law where they reside.

In our cohort, in three cases pES result influenced the couple' decision-making process, in one diagnostic case, in opting for TOP, in one nondiagnostic case to continue the pregnancy and in one inconclusive case with both LP and VUSs variants detected, in continuing the pregnancy.

In contrast, our systematic literature review showed that in papers describing the clinical impact of pES (n= 7/12, 58%), pES findings resulted influencing the decision-making process in more than half of cases (55,66%, range 26.32%-83.33%.).

The median TAT in our cohort for obtaining pES results was 13,4 days, a shorter time in comparison with literature data of our systematic review (available in 5/12 studies; 42%), in which the median delay resulted 27,37 days (range 17.35-42 days).

However, in Italy, legal time for TOP is during the 22nd GW, therefore maximum efforts are required to Genetic Laboratories to give results timely for couple decision making. In CNS anomalies, as previously discussed, it is even a harder effort in comparison to other anomalies, as often the diagnosis is around 22 GW, or later, making sometimes impossible for couple to wait for genetic results.

This actual scenario opens important ethical issues concerning the legal limit of TOP in Italy, leading women opting for a TOP, to make the decision without all the possible prognostic elements, such as a genetic result. In this regard, probably the enormous revolution in the availability of timely prenatal genome-wide diagnosis testing should be taken up by the Institutions.
Having a genetic diagnosis during pregnancy, allow also an appropriate genetic counselling for future pregnancies of the couples. In our cohort, the 5 diagnostic pES result, permitted to formulate appropriate recurrence risks, which varied between low risk for *de novo* variants (ID 3,4,5), to 25% in autosomal recessive condition (ID 1), up to 50% in the autosomal dominant condition inherited from the previously not molecularly characterized father (ID 2).

5.2.1 Phenotypes:

Patient phenotypes are extremely useful in narrowing candidate genes in untargeted sequencing such as ES and GS. However, in prenatal setting, fetal phenotypes of genetic diseases are often unique and at present are not completely described. A paucity of data is available about the prenatal manifestations of most Mendelian diseases, and many relevant articles are reports about single cases that are insufficient to understand the natural history of diseases.
Firstly, certain manifestations can occur prenatally but resolve or evolve postnatally, the intrinsic challenge of prenatal diagnosis is that fetal phenotypes may evolve over time and the occurrence of symptoms may be specific to the developmental stage (Mone et al., 2022).
In addition, new phenotypes or signs can be observed in more advanced gestational age, and phenotypes can resolve (*i.e.* cystic hygroma or hydrops fetalis) even in the presence of an underlying genetic disease. A recently published systematic review has shown that during routine third-trimester US, an incidental fetal anomaly can be found in about 1 in 300 scanned women. This not only includes anomalies missed at the earlier 20 weeks US scan but also abnormalities that can only be seen with fetal maturation, as in the case of many CNS anomalies, such as anomalies of cortical development, microcephaly, or hydrocephalus (Drukker et al., 2021).
In this regard, HPO descriptors are widely used to standardize clinical information, representing the more comprehensive database of abnormal human phenotypic features including signs, symptoms, laboratory test results, imaging findings, and other phenotypic abnormalities.
HPO is logically structured as an ontology and enables sophisticated algorithms that support combined genomic and phenotypic analysis in diverse clinical and research applications.
To date, almost all clinical genetics diagnostic tools leverage the HPO to encode and compute over patient features in the context of genomic variant classification.

However, from 2008, the majority of work on the HPO has been dedicated to describing postnatally observed phenotypes. With the advent of the application pES and pGS, an opportunity has arisen to apply HPO-based, phenotype-driven, genomic analysis to prenatal sequencing.

For this reason, a group of specialists in multiple disciplines related to prenatal medicine came together over a period of two years, adding 95 terms to the "Abnormality of prenatal development or birth" (HP:0001197) sub-hierarchy (for a total of 247) and 65 terms to the "Abnormalities of placenta or umbilical cord" (HP:0001194) category (Dhombres et al., 2022).

This valuable effort has certainly improved the HPO database, but specific issues should be still taken into consideration. For example, prenatal information comes from different imaging methods, such as 3D/4D US or Fetal MRI. These imaging methods offer indirect evaluation for anatomy and function, and HPO terms must be extended to include data from this kind of modalities. In addition, there is a clear difficulty in identifying some organs in the prenatal period (*i.e.* the skin) and some anatomical structures change significantly in the postnatal period (*i.e.* the lungs, the cardiovascular system), being impossible to assess functional anomalies such as seizures, developmental delay, or hearing loss (Dhombres et al., 2022). Also, dysmorphology signs are still limited to be assessed through 3D/4D US.

A further point is that terms to describe the "worsening" or "offset" of phenotypic features are still needed. Recently, the Global Alliance for Genomics and Health (GA4GH) is developing a suite of coordinated standards for genomics for healthcare (Rehm et al., 2021), including the *"Phenopacket",* created for sharing disease and phenotype information. *Phenopacket* provides additional context for the phenotypic abnormalities, beyond what could be provided by a simple list of HPO terms.

For instance, the age of onset of each feature is provided, and the fact that a particular sign was excluded on prenatal US examination is indicated. For prenatal features, the precise age of onset is often difficult to determine, and the "onset" field should be used to report the age of first observation.

As a first application of *Phenopacket* schema for prenatal medicine, a collaboration between the HPO team and the Fetal Sequencing Consortium (FSC) was recently started (Giordano & Wapner, 2022). The FSC includes more than 30 national and international sites involved in fetal sequencing whose members participate in bi-weekly calls to share data, challenges, and discuss new technologies relevant to sequencing in the perinatal period. The FSC has sequenced >3,000 affected fetuses or stillbirth probands, finding that 13–26% of fetuses with structural anomalies currently have a causative genotype using ACMG classification guidelines (Giordano & Wapner, 2022).

Participants in the FSC represent diversified aspects of prenatal genomics, including genetic counselors, clinical geneticists, molecular laboratory directors, clinical scientists, basic science and clinical investigators, maternal fetal medicine geneticists, prenatal imaging experts, neonatologists,

and perinatal pathologists (Giordano & Wapner, 2022). This effort has the aim to understand the genetic and phenotypic architecture of fetal anomalies and stillbirth and use this information to improve pre- and peri-natal, postnatal, and maternal care.

5.2.2 Filtering:

Trio inheritance filtering is chosen especially in prenatal diagnosis, to reduce the number of variants requiring analysis to expedite reporting. This analysis relies on the clinical status of the parents being unaffected and only filter variants of interest if they fit with the required inheritance pattern: *de novo* in the fetus for an autosomal dominant conditions, compound heterozygous or homozygous in the fetus for an autosomal recessive conditions and hemizygous for X-linked conditions with the mother carrier (or *de novo*).

In a recent literature review of a single center laboratory experience, on a cohort of 114 fetuses, this approach for analysis would have missed P/LP variants in nine cases (7.8%) (Chandler et al., 2022). In our own experience, 1/5 cases (ID 2) had an autosomal condition inherited from the father, without a pre-conceptional or peri-conceptional genetic counseling required by healthcare professionals and the possibility of a molecular diagnosis before performing the trio-based pES at 20 GW.

This scenario is not an exception, because often, referrals for fetal ES come from fetal medicine units, with limited input from clinical geneticists. Indeed, the father may often not be present at the time of the US scan. Conversely, examination of parents and ascertaining the family history with a three-generation pedigree in clinical genetics is crucial for a complete analysis. Many autosomal dominant conditions have variable expressivity and age of onset and parents may often be unaware of carrier status, therefore, this possibility should be included in parental pre-test counselling.

Another point to consider when applying filtering is that in some cases, also a dual diagnosis has been reported in literature in prenatal exome cohorts (Aggarwal et al., 2020) and therefore a potential second diagnosis should be considered in any analysis pipeline. In this regard, imprinted genes also need to be considered (Chandler et al., 2022).

The last point to consider is the possibility of parental mosaicism when applying filters to exome datasets: asymptomatic parents may be a somatic mosaic for a pathogenic variant with a variant allele frequency (VAF) high enough to be called by a variant caller, but these variants may then be filtered out if the inheritance does not fit that expected, based on the family history and clinical information available. It is therefore crucial that mosaicism is considered when designing pipelines and cut-offs for VAFs, although this will highly depend on the quality of the sequence data and probably will need manual inspection of parental sequencing reads for all "*de novo*" cases (Chandler et al., 2022).

This possibility may change substantially the recurrence risks for couples, as compared to a germline mosaic risk.

Finally, it should be considered that in cases with single pathogenic variants the second variant could be missed due to the presence of a CNV on the other allele (if the pipeline does not take this into consideration), gaps in the coverage of the gene, or if the second variant is not detectable by ES, for example, because it is deeply intronic. For cases that do not fit the expected inheritance pattern there could be also uniparental disomy or a *de novo* variant on the other allele (Chandler et al., 2022).

All these challenges, must be managed by laboratories with expertise in NGS technologies and expertise in prenatal diagnosis, requiring limited TAT times, to establish a timely molecular diagnosis.

5.2.3 Variants of Uncertain Significance

As opposed to panels that may include only well-defined disease-associated genes, pES and even more pGS, while increasing the predictive potential, will identify a larger number of uncertain variants in known genes as well as identify genes previously not known to cause human diseases.

At present, defining homogeneous criteria for reporting VUSs prenatally is a significant challenge. In most countries, prenatal molecular diagnosis of a known mutation is acceptable only for variants classified as P/LP according to ACMG guidelines (Richards et al., 2015).

However, the question of whether VUSs should be reported or not has not been resolved and standardized guidelines are not available, resulting in inhomogeneous conducts.

In the last Points to consider document (Monaghan et al., 2020), ACMG highlights how laboratories have different policies regarding prenatal VUSs report, even within the same country.

The first point to clarify concerns VUSs in genes related to the fetal phenotype. The benefit/risk ratio should be carefully assessed, since a VUS identified in an indication-relevant gene might impact current and/or future pregnancies, but uncertainties may cause parental anxiety without immediate benefit for decision-making (Ferretti et al., 2019).

ACMG suggests reporting VUSs in phenotype-fitting genes, especially for autosomal recessive conditions if a VUS is found in *trans* with a pathogenic/likely pathogenic variant (Monaghan et al., 2020). The last Updated Position Statement of ISPD on prenatal genome-wide sequencing (Van den Veyver et al., 2022), recommends that *result reporting from sequencing data on fetal samples is best focused on pathogenic and likely pathogenic variants in genes that are relevant to the fetal phenotype. It is recognized that some laboratories may report variants of uncertain significance in strong candidate disease genes for the fetal phenotype, for example, in an autosomal recessive gene which is relevant for the fetal phenotype, when a pathogenic (or likely pathogenic) variant is inherited from*

one parent along with a variant of uncertain significance from the other parent. This should be addressed in pre-test counselling, and in these situations, expert genetic post-test counselling is highly recommended (Van den Veyver et al., 2022).

However, the choice of reporting VUSs is currently ascribed to the single laboratory policy. In a recent review of a single laboratory experience (Chandler et al., 2022), authors stated that in most cases, only P or LP variants explaining the fetal phenotype were reported. Only in few selected cases, VUSs which may explain the phenotype and only require a single piece of evidence to be upgraded, the so called *"hot" class 3s*, might be reported. The multidisciplinary team for discussion is crucial to ensure the variant fits the fetal phenotype and, in some cases, may allow upgrading to P or LP variants. Sometimes, VUSs could be included on the report to highlight the need for further prenatal surveillance and postnatal investigations if appropriate (Chandler et al., 2022).

In our own experience, laboratory policy reports all the detected VUSs in genes related to the fetal phenotype, leading to an impressive ambiguous result rate up to 40%. In fact, in 6/15 cases of our cohort, multiple VUSs in single cases were detected. In one case both a LP variant and VUSs were detected and were interpreted as only partially explicative of the fetal phenotype by the multidisciplinary team. In our recent systematic review focused on all kinds of US anomalies, in unselected fetuses for fetal anomalies (Group A) the overall VUS rate was 8.32% (7.94–8.69%), a relevant percentage in comparison to the total diagnostic yield (Mastromoro et al., 2022).

Specifically concerning CNS anomalies, from the literature systematic review performed in this phD thesis, only five out twelve papers reported inconclusive results, explicitly declaring the number of VUSs, therefore we did not score a specific rate due to too low number of papers reporting these findings. In fact, in many countries, such as in France according to a national law, VUSs are not reported in prenatal diagnosis (Heide et al., 2020).

Correctly interpreting these variants takes on increasing importance to clinical management. This requires not only determining the pathogenic potential of the specific variant but also determining whether the variant is causative of the known fetal anomaly (Giordano & Wagner, 2022). Pathogenicity of specific variants is usually determined by the laboratory using standardized protocols and available databases. However, determining the association between genomic variants and fetal phenotype requires the input of maternal fetal medicine experts, imagers, developmental biologists, clinical geneticists, clinical scientists, and genetic counselors where available.

In FSC experience, during internationals calls, cases are formally presented with slides describing the clinical scenario, including the phenotype, and presenters are encouraged to present related HPO terms. Genomic findings are presented in detail along with the details of the structure and function of

the involved gene, specific variant, existing knowledge of the variant including literature database review, and the center's initial interpretation (Giordano & Wapner, 2022).

During my phD course, I spent 10 months in Switzerland, at the Division of Medical Genetics, Geneva University Hospital. I was part of the CGEM (Centre de Génomique Médicale pluridisciplinaire), in which *"Genome Boards"* meetings were organized weekly to discuss molecular variants detected by ES or GS (the latter in research selected cases). Every week a different Genome Board was scheduled with an annual calendar for each specialty (neurology, hepatology, immunology, cardiology, endocrinology, neuropediatrics) (Fig.19). Specific Prenatal Genome Boarding were scheduled upon request weekly or every two weeks.

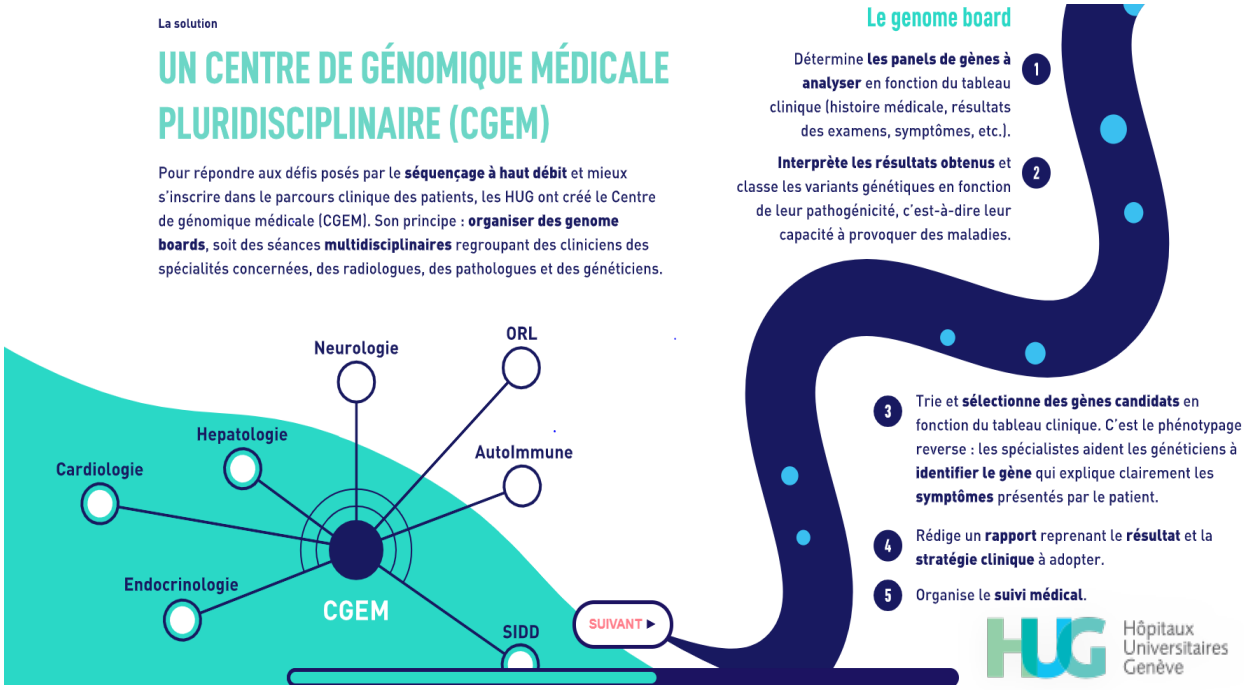

Fig.19: Genome Boards Organization at Geneva University Hospital

The participants at the meetings were the referral clinicians according to the daily-specialty, clinical geneticists, radiologists, and anatomo-pathologists if required, as well as the bioinformatician and molecular biologists who performed the in-silico analysis. All members had the possibility to participate in person or from remote connection, as we were during the COVID pandemic in 2021. During the meetings a brief case report was presented to all the participants, including a projection of the pedigree and family history, relevant instrumental data and/or radiological images and/or photos. Interestingly, in this process, all variants were firstly evaluated by three biologists independently

("triple blind-analysis") only based on the biological characteristics of the variant (i.e. frequency, type of variant, previous description in international databases, functional studies available), and classified by a color based criterion (green: interesting variant, yellow: possible interesting variant, red: false variant or sequencing problems *etc.*), then the selected variants were evaluated by the clinical geneticist, to establish the possible match between genotype and phenotype and to study the available literature. Only after these steps, each case was discussed during the Genome Boards meetings and variants classified according to ACMG guidelines (Richards et al., 2015), with a multidisciplinary agreement in reporting or not reporting VUSs. In my 10-month experience, the majority of VUSs was not reported, unless in the presence of very high evidence to be possibly re-classified in LP or P variants in the nearest future.

In fact, in choosing if reporting or not VUSs, it should be considered that variant classification may evolve over time as new evidence emerges (Ferretti et al., 2019).

In this perspective, postnatal sequencing data access is appealing and underestimated. Fetal results are normally included in the mother's records, making data-retrieving difficult for child follow-up (Guadagnolo et al., 2021). It has been suggested that pES data reanalysis should be considered if a new phenotype emerges after birth or a future pregnancy is planned, but the exact time has not been clarified yet (Mellis et al., 2018). In pediatric population has been demonstrated that ES data re-analysis boosts after 12 months (Ewans et al., 2018). It is also debated if clinicians or laboratories should be primarily responsible for such re-analysis (Mellis et al., 2018).

Inconclusive results could be also linked to variants in a "candidate gene" (gene with limited evidence of disease association). ACMG statement suggests classifying variants in candidate genes no higher than a VUS and specify the policy during consent process (Monaghan et al., 2020).

However, it should be considered that fetal evaluation has the additional complexity of identifying previously unrecognized *in utero* phenotypes and genotypes, as well as genotypes that are rarely, if ever, seen in children and adults due to their lethality (Giordano & Wapner, 2022).

An example fitting this scenario, is the *BICD2*-related prenatal phenotype (SMALED2B MIM 618291) of case ID 4. We described the first prenatal molecular diagnosis of a severe BICD2-opathy, published in early 2021 (Marchionni et al., 2021), and in the following months other prenatal cases were reported, expanding the features of prenatal phenotypes.

We firstly presented the case at the European Society Human Genetics Conference online due to the Covid Pandemic in 2020, and an English Genomic Laboratory contacted us for a similar case.

Few months later, another fetus affected by AMC was referred to Medical Genetics Laboratory of Ospedale Pediatrico Bambino Gesù and diagnosed with a BICD2opathy. In addition, in the recent work of Chandler et al., authors reported another prenatal case with a *BICD2* variant who presented

with hydrops (Chandler et al.2022) that, at the time of initial reporting, was not a described feature of this condition. However, they report that the following week a paper was published linking hydrops to a *BICD2* pathogenic variant, allowing the upgrade to LP variant (Al-Kouatly et al., 2021).
In this perspective, it is likely that many of the causative genes for the most severe *in utero* phenotypes have yet to be fully cataloged in the OMIM database due to the rarity of many of these conditions in pediatric cases.
Stanley et al. recently highlighted that in a cohort of 246 stillbirth probands evaluated using ES, there was an enrichment of loss of function variants in genes intolerant to such variation in humans and these were concentrated in genes not previously associated with human disease (Stanley et al., 2020).

5.2.4 Incidental and Secondary Findings

More complex problems concern variants unrelated to the fetal indication, such as SF. For postnatal ES secondary findings, the ACMG actually recommends reporting pathogenic/expected pathogenic variants in 78 actionable genes, causing highly penetrant disorders for which interventions preventing/reducing morbidity and mortality are available (Miller et al., 2022).
This document does not provide specific indications for SF and IF in prenatal diagnosis (Miller et al., 2022). The previous ACMG statement (56 genes) recommended to include children, explicitly excluding fetuses (Green et al., 2013). A subsequent revised document in 2015, suggested to offer an opt-out option (ACMG Board of Directors, 2015) and in 2020, the last ACMG statement published, only focused on prenatal sequencing, confirms the opt-out option, recommending reporting SF if parents consented (Monaghan et al., 2020).
However, the possible compromission of the individual's future possibility to decline receiving such results, if the parents consented, is largely discussed (Ferretti et al., 2019). The last updated Position Statement of ISPD (Van den Veyver et al., 2022) recommends that *the option for inclusion or exclusion of SF in the fetal and parental sequence should be addressed.* In addition, it specifies that *each parent should consent separately to inclusion or exclusion of SF for their own sequencing results* and *fetal SF with a moderate to severe childhood condition should be discussed for inclusion/exclusion consent.* Interestingly, the recommendation also includes the opposite situation, in which only a panel of genes and not a wide-genome sequencing approach is used.
In that case, ISPD specifies that when *using a panel approach to analysis, parents should be advised that SFs are not looked for.*
A recent survey on genetics professionals' attitudes towards pES highlighted that for most participants the definition of "actionable results" is different in the prenatal setting due to the option for termination (Brew et al., 2019).

It should be underlined that SF and IF are not synonymous, even if in literature often are used as interchangeable terms (Guadagnolo et al., 2021). IF include variants unrelated to primary test indication and not included in the ACMG secondary findings list. These include IF in genes associated with neurodevelopmental disorders, intellectual disability, or metabolic conditions that are highly penetrant and are known to cause moderate to severe childhood disorders. These conditions in prenatal diagnosis may present without US findings. Currently the ACMG statement recommends reporting only high-penetrant pathogenic variants known to cause moderate/severe childhood onset disorders (Monaghan et al., 2020). The last ISPD Position Statement (Van den Veyver et al., 2022) specifies that *each parent should consent to inclusion or exclusion separately and Fetal IF should be discussed for inclusion/ exclusion during consent.* For IF, ISPD also recommends that when *using a panel approach to analysis, parents should be advised that the risk of detecting IFs is small and will be restricted to genes on the panel used*.

Additional findings such as non-paternity or consanguinity are also detectable in pES, and parents should be aware before testing is undertaken that these may be identified because these results could provide couples and families with unwanted information (Ferretti et al., 2019).

In trio-based analysis, SF and IF may be searched for and detected in the parental as well as in the fetal genotypes. ACMG statement (Monaghan et al., 2020) recommends laboratories to establish clear policies regarding whether IF report is limited to fetal variants or parents are also included (irrespective of fetal inheritance).

Of note, the laboratory's analysis process could be set to limit the assessment of parental variants to only those present in the fetus. In our cohort we did not find IF or SF findings, however the possibility to obtain such results after sequencing was previously discussed with parents, giving the chance to opt-in or opt-out during the consent process. In the systematic literature analysis, SF/IF findings were reported in one paper only (Yang et al., 2022), reflecting the managing difficulties for such results, especially in prenatal diagnosis. In addition, it should be borne in mind that pES is usually proposed for US single or multiple anomalies, couples already presenting apprehension for the possible conditions underlying these anomalies (Guadagnolo et al., 2021).

They often lack sufficient time to reflect about the potential amount of information available after pES and to elect how much they want to know. Reporting inconclusive or SF/IF in this context is extremely delicate and offering psychological support should be considered (Richardson et al., 2018).

A possible approach to reduce interpretative and ethical issues is to report only variants related to the specific indication or pregnancy-/delivery-relevant conditions or suggesting fetal therapies (The Lancet, 2019). In this perspective, parents could opt for a secondary report to disclose findings relevant to postnatal care.

5.2.5 Ethics:

Current challenges in pES counseling also concern the importance of adequate consent, a long-lasting debate in prenatal diagnosis (Quinlan-Jones et al., 2016).

In the era of prenatal genomics additional issues emerge, due to the unprecedented quantity of detailed genetic data to be reported or retained, as discussed above. The process of consent requires patients to achieve a good understanding before decision (Horn & Parker, 2018) and professionals should provide available and manageable information (Westerfield et al., 2014) to consultants from all educational and cultural backgrounds (Best et al., 2018). This is not always feasible, as for couples with low school-education, for whom also the concept of DNA could be difficult to manage or when language barriers do not allow an appropriate understanding (Guadagnolo et al., 2021).

Returning much information after pES could influence decision-making about pregnancy termination (Best et al., 2018) or the parents' attitude towards their child (Horn & Parker, 2018), being informed about the risk of developing signs or symptoms of a genetic condition.

The emotional state of the consultants, who often receive counseling immediately after the detection of the US anomaly, must also be considered in this scenario. Incertitude about disease severity, the probability of presenting some symptoms or not and the emotional tool of a genetic condition are always present, this incertitude may be increased in prenatal diagnosis and should be clearly presented to parents (Richardson & Ormond, 2018).

The resulting confusion and the disruption of the expectation of a healthy fetus can make it difficult to understand genetic implications and give true informed consent for testing (Richardson & Ormond, 2018). It is also essential to provide the basic communicative skills to the other health professionals, as fetal medicine is a multidisciplinary work, involving gynecologists, radiologists, and pediatricians. Ideally, the communication should take place in a safe environment and in the presence of the whole team but unfortunately, this clashes with practical concerns (Mastromoro et al., 2022).

Healthcare professionals should be aware of the stress caused to parents by an atypical fetal exome result and ensure appropriate genetic counselling and psychological support (Guadagnolo et al., 2021). Various studies have demonstrated that both ethical and practical concerns on topics such as consent and data interpretation exist among practitioners regarding the use of pES (Horn & Parker, 2018; Quinlan-Jones et al., 2016). A recent study evidenced a difference of opinion about the best timing for obtaining patient consent among healthcare professionals. When senior practitioners were surveyed on the best time to obtain consent for pES, 33% answered that it is during the initial genetic consult when diagnostic testing is offered and 48% answered that it is after nondiagnostic results of karyotyping and CMA. In contrast to all practitioners, 71% of the practicing genetic counselors and

students surveyed answered that, the best time to obtain consent for pES, it is after the nondiagnostic results of karyotyping and CMA and 25% answered that it is during the initial genetic consult when diagnostic testing is offered (Johnson et al., 2021). The same study highlighted the lack of consensus among the medical and diagnostic laboratory community regarding the clinical utility and appropriate use of pES. Attitudes toward the use of pES statistically differed by type of participant and level of training. Overall, practicing genetic counselors and physicians were more selective in their recommendations for pES than laboratory scientists, and practicing physicians were more selective in their recommendations for pES than medical students (Johnson et al., 2021).

In this regard, practicing physicians may have a better understanding of the ethical and practical concerns associated with pES. This study demonstrates the need for increased education on pES for the medical community, including the importance of genetics education in the preclinical years of medical school, and the need to better disseminate preexisting guidelines on the use of pES (Johnson et al., 2021).

As a final remark, negative results (no variants disclosed) from genetic tests could be mistakenly interpreted by the consultants as reassuring findings of a normal outcome, but it should be discussed and clarified that a non-diagnostic analysis reduces the risk of a genetically determined condition without ever being able to exclude it.

It could be reassuring during pregnancy, but the "temporality" should be clarified beforehand, due to the evolving fetal phenotype and possible postnatal appearance of further anomalies (Guadagnolo et al., 2021). In fact the significance of a negative result should be still being investigated and long term follow-up of born children with pES nondiagnostic results, is of outmost importance to provide accurate prenatal counseling when all genetic analyses are inconclusive.

5.3 Future perspectives

According to the last ISPD statement, there is currently no evidence supporting pES routine testing (including upon parental request) on fetal tissue obtained from an invasive prenatal procedure for indications other than fetal anomalies (Van den Veyver et al., 2022). However, in literature some papers exploring the possibility of pES use in clinical practice in pregnancies without structural anomalies are available. In 2022, Vaknin et al., performed pES in 210 pregnancies without abnormal fetal ultrasound findings (subcategories 2a: low risk pregnancies with minor US findings and 2b: normal pregnancy surveillance). In this group, 6/210 (2.86%) cases were found to have P/LP variants [5/50 in (2a) and 1/160 in (2b)], showing an unexpected rate of genetic diagnosis on pES performed in apparently normal pregnancies or with minor US anomalies (Vaknin et al., 2022). This result will possibly be confirmed by other studies ongoing, opening the question on pES application also in the

absence of major fetal anomalies. Careful attention should be used in this scenario, especially for the management of inconclusive findings, such as VUSs.

Another possible future scenario is the substitution of karyotype/CMA plus pES analysis with pGS analysis. As previously discussed, GS allows the sequencing of an individual's whole genome, potentially identifying more genetic variations than any other approach, raising further questions about interpretation and reporting of the variants. Two different strategies are currently available. The first one is the high-coverage GS, which explores anomalies ranging from chromosomal to CNVs and SNVs in a single analysis. A recent study applied a trio-based pGS to a heterogeneous population of 102 fetuses affected by structural and dynamic anomalies with an overall diagnostic rate of 17.6% (Zhou et al., 2021). Another study applied pGS on a prospective cohort of 37 singleton fetuses presenting with structural abnormalities, identifying SNVs in 5 fetuses (14%). Importantly, pGS also identified all pathogenic variants reported by clinical microarray (2 CNVs, 5%), allowing a definitive diagnosis (SNVs + CNVs) in 19% of fetuses with structural anomalies (Wang et al., 2022).

Another study applied singleton pGS to a cohort of fetuses presenting with increased NT (nuchal translucency) and showing an overall diagnostic yield of 32% (Choy et al., 2019). The diagnostic rate resulted higher compared to the previous pGS study, possibly due to the smaller sample size and to the lower heterogeneity of this second cohort. The second approach is low-coverage genome sequencing (LC-GS) (Wang et al., 2018; Wang et al., 2020; Chau et al., 2021; Walker et al., 2019; Huang et al., 2021). Strong evidence supports that this approach, identifying gross and submicroscopic CNVs and chromosomal anomalies, could be applied as an alternative to CMA in prenatal diagnosis (Wang et al., 2020; Chau et al., 2021), requiring less DNA (thus reducing the need for cell culture) and performing better in regions with lower CMA probes density. The diagnostic yield of LC-GS resulted lower than high-coverage GS, as expected due to the different sensitivity, displaying less marked increase in the diagnostic yield than the gold standards approach (Wang et al., 2020), with a more noticeable increment if compared to karyotype (Huang et al., 2021).

In contrast, parallel application and comparison between pGS and the respective CMA results highlights a significantly higher diagnostic yield of the first approach (Zhou et al., 2021; Yu et al., 2021, Wang et al., 2021), which is consistent with the pGS ability to also detect SNVs.

Finally, the overall VUSs number is consistently higher in the pGS cohort that reported this result (Choy et al., 2019; Wang et al., 2022) than in the LC-GS studies (Wang et al., 2018; Huang et al., 2021; Wang et al., 2022) (38% and 19% vs. 1.4% and 3.8%), potentially imputable to the higher number of variants detected with the first approach. These available studies demonstrate that pGS has the potential of replacing multiple consecutive tests, including microarray, gene panels, and pES, to provide the most comprehensive analysis in a timely manner necessary for prenatal diagnosis.

In this regard, at present, there are preliminary prenatal (Zhou et al., 2021; Yu et al., 2021, Wang et al., 2021), and postnatal (Lindstrand et al., 2019, Stranneheim et al., 2021) clinical data to demonstrate similar performance of GS in comparison to ES plus CMA concerning diagnostic yield and potential for lower cost for GS, which would depend on sequential or concurrent ES plus CMA application.
In fact, in postnatal setting, using ES as a first- or second-tier test (e.g., after CMA or targeted testing) yielded more diagnoses at a lower cost than using ES only after extensive standard testing (e.g., large sequencing panels and/or multiple testing approaches) or using standard testing alone.
With the anticipated further declines in cost, early use of genome-wide sequencing should continue to enable more timely diagnosis for patients with unexplained developmental delay or multiple congenital anomalies in postnatal setting (Manickam et al., 2021) and probably in prenatal setting too, opening a possible larger application soon.
In addition, the possibility of ES and GS data re-analysis is stimulating, along with newborn follow-up. This approach requires NGS data accessibility after birth, and coordination between professionals and Institutions to avoid repeating genetic tests.
Different approaches have been proposed even by ACMG, such as periodical reanalysis of negative results, or targeted re-analysis for cases with evolving phenotype (Ewans et al., 2018, Manickam et al., 2021). It has been suggested that pES data reanalysis should be considered if a new phenotype emerges after birth or a future pregnancy is planned, but the exact time has not been clarified yet and it is also debated if clinicians or laboratories should be primarily responsible for such re-analysis.
Re-evaluation of the evidence base for ES and GS should be done at regular intervals given rapid technological advancements leading to improved diagnostic yield, variant interpretation, and reduction in cost. The exact interval of this approach should consider the resources required and should consider significant organization efforts and world-wide guidelines to produce homogeneous data. Also negative, nondiagnostic results should be revaluated. Long-term follow-up of born children is of utmost importance to provide accurate prenatal counseling when pES results show no detectable abnormalities. In addition, a negative result could be useful to help exclude (or make less likely) genetic conditions that may have prognostic implications (e.g., increased risk of seizures in CNS anomalies) or other clinical management (e.g., contraindication to anesthesia/surgery).
In coming years, in order to optimize pES (and future pGS) reliability and reduce interpretative issues, a deep fetal phenotyping would be advisable. ES and GS data from large control prenatal populations similar to the genome aggregation database (gnomAD) for postnatal cases, still does not exist, making it difficult variant interpretation in prenatal diagnosis (Giordano & Wapner, 2022).
Although broad guidelines regarding variant interpretation and gene-disease associations exist, the currently available data sources do not specifically differentiate fetal from childhood and adult

findings and do not have sufficient information on the fetal phenotype, the gestational age at presentation, and unique in utero findings (Giordano & Wapner).

Therefore algorithms, software and bioinformatic tools should be specifically recalibrated for fetal signs, and fetal variant databases, equivalent to postnatal databases such as ClinVar or HGMD, improved. In this regard, great expectations are placed on integration, aggregation and analysis of phenotypic and genotypic data retrieved from the FGC centers. This collaboration is planned to create the Terra Platform (https://terra.bio/), a cloud-based data ecosystem enabling processing and analysis of genomic data. Integrating phenotype data into analysis in Terra is currently limited, and a goal is to build-out the ability to easily store and run analysis using Phenopackets and associated tools in Terra being accessible to researchers and clinicians worldwide (Giordano & Wapner, 2022).

In conclusion, this large overview highlighted current evidence on advantages and disadvantages of pES (and pGS) application and future directions. These approaches are revolutionizing prenatal medical genetics and the management of fetal detected US anomalies.

6. CONCLUSIONS

In the early 2010s, CMA became a standard prenatal diagnostic tool and new themes emerged about what constitutes a diagnostic finding, an uncertain finding, a clinical actionable or incidental finding. Over the last 5 years, pES, and more recently pGS analyses, have proven valuable in determining the genomic causes of many fetal structural anomalies and stillbirths.

In addition, prenatal sequencing has also been extremely helpful in elucidating and expanding fetal phenotypes of known disease-causing genes previously related to postnatal phenotypes only.

However, the application of pES raises several challenges and optimized indications, detection rates in different fetal anomalies, and interpretation of variants pathogenicity are still under investigation.

The aim of this study was to assess the incremental diagnostic yield of trio-based pES after karyotype and CMA inconclusive result in CNS fetal anomalies.

In our single-centre cohort of fetuses presenting with apparently isolated and not isolated CNS anomalies, pES showed a high incremental diagnostic yield (33%) over karyotype and CMA alone.

The systematic review and meta-analysis performed on published cohorts of fetuses affected by CNS anomalies confirms this result. The pooled incremental diagnostic yield estimate over karyotype and CMA resulted 36% (95% C.I.: [28%;44%]) in all CNS anomalies, 22% (95% C.I.: [15%;31%] in apparently isolated CNS anomalies, 32% (95% C.I.: [22%;45%]) in CNS-only related anomalies (one or more) and 45% (95% C.I.: [37%;53%]) in non-isolated anomalies (either ≥ 2 anomalies in CNS, or extra-CNS).

In conclusion, the pooled results showed a substantial diagnostic improvement in performing genome wide sequencing analysis over standard and molecular cytogenetics, supporting the proposal of offering pES earlier in the diagnostic route of CNS fetal anomalies.

At present it is advisable to perform pES in parallel with CMA, bearing in mind that, in the near future, the CMA plus pES approach might be replaced by pGS analysis as first-tier test.

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