
Title: Pediatric and developmental pathology
ArticleTitle: In a genomic era, placental pathology still holds the key in the nondysmorphic stillbirth
ArticleAuthor: Campbell
Vol: 21 No: 3 Date: 2018
ISSN - 10935266, 16155742;

Original Article

Pediatric and Developmental Pathology
2018, Vol. 21(3) 308–318

DOI: 10.1177/1093526617733373
journals.sagepub.com/home/pdp
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In a Genomic Era, Placental Pathology Still Holds the Key in the Nondysmorphic Stillbirth

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Abstract

Objective: To explore the relative utility of genetic testing in contrast to placental pathology in explaining causation of death in the structurally normal stillborn population.

Methods: A retrospective review of a structurally normal stillborn infant cohort in South East Scotland between 2011 and 2015, defined by death at or after 24 weeks of gestation. We reviewed pathology reports and collected demographic data on cases. This information was collated with genetic test results (quantitative fluorescent polymerase chain reaction and microarray analysis) and placental pathology to create a database for analysis.

Primary Results: Within the structurally normal population (n = 131), there were 125 genetic tests performed and 11 abnormal results. Sixty-six microarray analyses were performed, and 2 (3%) of the results were thought likely to reflect cause of stillbirth (1 case of incomplete trisomy 4 and 1 case of deletion of chromosome Xp in a female). Analysis was significantly limited in 2 cases as parental samples were not available. The placental pathology was available in a total of 129 cases; significant findings were identified in 100 cases; 79 (61%) showed changes that were considered to have caused death (including cord "accidents"), and a further 21 (16%) showed findings likely to influence the management of subsequent pregnancies.

Conclusions: We reaffirm the utility of placental examination in the investigation of stillbirth. In cases of nondysmorphic stillbirth where placental pathology does not explain the cause of stillbirth, microarray analysis of fetal DNA can add further diagnostic information in 3% of cases but can add further diagnostic confusion, and it is important that parental bloods are taken to minimize this risk.

Keywords

intrauterine growth restriction, microarray, placenta, stillbirth, postmortem, nondysmorphic

Introduction

In the United Kingdom, 1 in 200 to 240 pregnancies end in stillbirth, and the reduction in incidence has been slower than the reduction in maternal mortality or childhood deaths up to 5 years of age. One study showed that when the placenta was considered alongside a detailed autopsy, the exact cause of death could be identified in 57.9%[1] of the cases. A systematic review of placental pathology reported in association with stillbirth showed that the proportion of stillbirths attributed to a placental cause ranged from 11% to 65%, and classification systems that permitted the inclusion of placental findings reported higher rates of placental causes and fewer unexplained stillbirths.[2]

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Genetic causes have been implicated in approximately 25% of stillbirth cases,[3] and these occur more frequently in cases where there are defined structural abnormalities. Microarray analysis allows assessment of the genome at higher resolutions than traditional karyotyping. Placental pathology has been shown to occur more frequently in the stillborn population and may give insight into causation and thus prevention.[4,5] It is unclear what impact new genetic testing methodologies will have on stillbirth diagnosis and counselling, and it is important to remember that in the majority of stillbirths, an explanation is more likely to come from detailed autopsy and placental pathology. For developing countries, genetic testing can be expensive; therefore, routine placental analysis may offer a more cost-effective means of assessing stillbirth. The objective of this study is to evaluate the utility of microarray in providing an explanation for nondysmorphic stillbirths in a routine autopsy setting.

Genetic laboratory investigations such as microarray analysis and quantitative fluorescent polymerase chain reaction (QF-PCR) may increase the diagnostic yield in the assessment of fetal dysmorphology. However, some abnormal results require careful review given their potential uncertain significance and a multidisciplinary team discussion to ensure the information is disseminated to the family in the best possible fashion.

Obstetric teams should ideally include specialists well versed in fetal anomaly and placental pathology to provide families with the best possible information about the cause of their pregnancy loss and their options for future pregnancies. A diagnosis in stillbirth is important to parents as it forms the basis of counselling to assess recurrence risk and the possibility for intervention in subsequent pregnancy.

Numerical and structural chromosomal anomalies are a major cause of stillbirth and perinatal morbidity and mortality.[3–5] Our center has been consistently using microarray studies alongside QF-PCR for aneuploidy detection on postmortem fetal tissue since mid-2010.

Prior to this, conventional G-banding karyotyping was performed which could detect chromosomal imbalances of greater than 10 Mb (10 million bases). Best practice guidelines for testing solid tissue samples require a success rate of at least 65% in contrast to 95% for fresh blood samples. This implies that a higher failure rate is inevitable with postmortem tissue. This is most likely because of poor quality tissue samples, high infection rates associated with pregnancy loss, and subsequent poor culture success. Microarray analysis does not require culture and is therefore more robust although failure may still occur, usually secondary to DNA damage because of severe fetal maceration. It detects changes in copy number (deletions/duplications) across the genome. Microarray analysis is most commonly applied to the investigation of children with developmental abnormalities, including malformations or developmental delay.[6] In recent years, the technique has been used to analyze fetal tissues following loss or termination for structural abnormality.[7–9] The resolution of current microarray platforms allows detection of copy number variations (CNVs) which would otherwise remain undetected by traditional G-banded karyotyping.

In this article, we examine the value of detailed postmortem and placental examination when assessing causation of stillbirth in the normal fetus and compare this with microarray analysis. Stillbirth in our cohort is defined as death at or beyond 24 weeks of gestation in keeping with consensus on the threshold of viability.

Materials and Methods

Postmortems of stillborn infants in South East Scotland are carried out in a single pathology department by 2 fetal pathologists. A retrospective review of fetal postmortem reports and genetic test results was performed. Data were collated on all stillbirths in the region and cross-checked by the authors. Calculation of individualized growth potential centiles (Customized calculator—The Perinatal Institute)[10,11] was performed retrospectively and applied to the cohort. An adjustment was made in each case to allow for estimated timing of the antenatal demise and postmortem examination. In all cases, we reevaluated the gestational age using foot length measurement which has been shown in many studies to correlate well with gestational age[12–14] and took account of the degree of maceration before using the customized centile chart.[15] The average birthrate for the region is 14 500 per year, and stillbirth rate is between 3.1 and 4.9 per 1000 deliveries which is in keeping with the national average for Scotland of 5.1 per 1000 in 2011 and 4.7 per 1000 in 2012.16,17

Inclusion Criteria

- Stillbirth that occurred between January 2011 and December 2015.
- Nondysmorphic stillborn infants delivered following 24 completed weeks of gestation from last normal menstrual period to term and beyond.
- Neonatal deaths within 2 h of birth. This includes failed resuscitation attempts.

Exclusion Criteria

- Cases reported to the Procurator Fiscal (Scotland).
- Cases with no consent for genetic testing included in autopsy authorization.
- Cases where anomalies were diagnosed antenatally or at postmortem.

DNA Extraction and Quality Check

The fetal gonad was sent for analysis. Where samples failed, we recorded condition of the fetus and any significant delays in transport to the laboratory for the analysis.

DNA was extracted from the fetal tissue using a Qiagen EZ1 robot after homogenization of tissue using Bertin technologies Precellys 24.

Quantitative Fluorescent Polymerase Chain Reaction

QF-PCR was performed using the Elucigene QST-V2 and QST*R pregnancy loss kit.

Array Comparative Genomic Hybridization Platform

Over the 5-year period included in this study, 2 different array platforms were used: NimbleGen (135K, CGX, and ISCA slides) and Affymetrix Cytoscan 750K arrays. Both platforms provide high-resolution genome-wide coverage, with at least 1 oligonucleotide probe per 30 kb backbone coverage with denser probe coverage in target regions of interest, eg, Online Mendelian Inheritance in Man (OMIM) genes. Both designs were performed in a similar manner for the detection of CNVs. The Affymetrix Cytoscan platform also includes single nucleotide polymorphism probes which allow the detection of loss of heterozygosity. For tissue investigations, we initially set the minimum size threshold for a copy number change at 1 Mb (1000 kb), partly due to performance issues with the quality of DNA derived from postmortem tissue samples and as a compromise between generating useful results and variants of unknown significance. The Affymetrix system has been demonstrated to be more robust and proved much less susceptible to problems with low-quality DNA samples, and therefore, lower calling thresholds have been applied. Affymetrix was introduced in April 2013, and a total of 50 (55%) out of 91 microarrays were carried out using this platform.

To analyze which genes may be present in the region of duplication or deletion, the results were compared to the DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) database. The database has links to OMIM which is a database housed in the United States National Center for Biotechnology Information and where possible links human disease to genes in the human genome (OMIM morbid genes).

Interpretation

Where abnormalities were detected, the case was reviewed with analysis of the phenotype at a multidisciplinary meeting attended by cytogenetic clinical scientists, clinical geneticists with expertise in fetal medicine and dysmorphology, and a fetal pathologist. Parental samples were then requested, where appropriate, to ascertain if the abnormality was de novo or inherited. This allows the content and effect of the CNVs to be considered in the context of the fetal pathology findings. The result is then defined as pathogenic of uncertain significance or benign.

Placental Histology

The placenta was examined fresh, and blocks were taken in line with the protocol outlined in the Amsterdam consensus statement.[18] Two samples of umbilical cord, 1 sample of membranes, and 3 random blocks of parenchyma were taken for histological evaluation. Any lesions noted macroscopically were sampled in addition to these standard blocks. Placental pathology was broadly categorized into hemorrhage, infarction, acute infection, villitis of unknown etiology (VUE), chorangiosis, and massive perivillous fibrin deposition (MPVFD) in line with Redline's Classification of placental lesions.[18,19] Assigning cause of death to placental pathology can be subjective and requires detailed clinic-pathological conference. In line with Man et al.,[20] we assigned death using criteria from a number of the recognized systems[21] including Causes of Death and Associated Conditions (CODAC). We also made reference to Redline's Classification of placental lesions[13] and the postmortem findings.

Cause of death was attributed to hemorrhage if there was a positive and significant Kleihauer test or significant crater formation with adherent clot. Presence of these features was taken as evidence of catastrophic feto-maternal hemorrhage or retroplacental hemorrhage; it was also regarded as significant if it could be temporally related to the death. The significance of placental infarction was assessed by the degree of compromise noted in the background, ie, features of significant maternal vascular malperfusion/ischemia with extravillous trophoblast proliferation or infarction >50% by volume associated with intrauterine growth restriction (IUGR; birth weight <10th centile). Zones of infarction <50% were considered as leading to compromise but not necessarily causing the ultimate demise of the infant in utero unless there were areas of full thickness infarction associated with disruption of the basal plate and acute hemorrhage. Acute infection was regarded as causative if there was a clear evidence of infection within the lung parenchyma at autopsy. VUE and occasional foci of FTV were regarded as contributory but not the definite cause, although it may inform care of future pregnancies.

Results

Over 5 years, there were 158 cases of stillbirth that underwent autopsy, of which 131 (83%) had no significant developmental anomalies and thus met criteria for

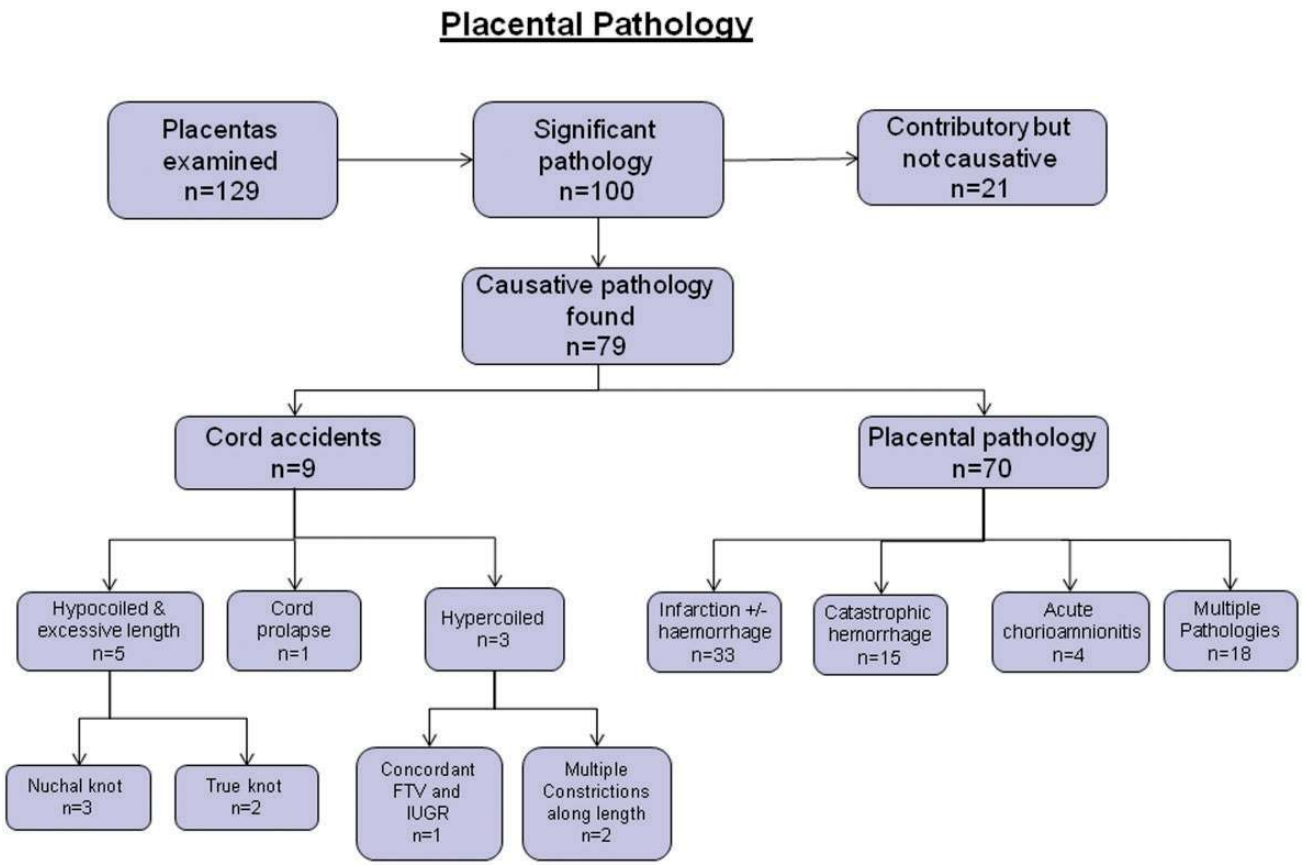


Figure 1. Placental pathology.

inclusion in this analysis. Please see Figure 5 for details of population gender, habitus and gestation. In 129 of these (98%), the placenta was also available for diagnosis. The cause of death was attributed to placental pathology or cord abnormalities in 79 cases (61%). For further details, refer to Figure 1. Nine deaths were thought to relate to abnormalities of the umbilical cord—1 cord prolapse (with normal coiling), 3 nuchal cords, and 2 true knots associated with color changes—these 5 cords were hypocoiled and of excessive length which was felt to increase the significance of the findings.

In 70 cases, the cause of death was explained by the placental pathology—33 placental infarction with or without other findings such as hemorrhage, background ischemia, or FTV; 15 isolated catastrophic hemorrhages associated in time with the demise; and 4 acute chorioamnionitis. The reminder showed multiple pathologies including chorangiosis and MPVFD. As might be anticipated, placental pathology causing death in utero was identified more frequently in cases of IUGR (46/66, 70%) than in cases with no evidence of IUGR (24/63, 38%).

In a further 21 cases, the findings in the placenta were thought to have contributed significantly to the in utero compromise and to have played a part in the death though the death could not be wholly assigned to the pathology. In total, therefore, excluding the cord "accidents" detailed earlier, about which little can be done, pathology which would inform the management of subsequent pregnancies was identified in 91 cases (71%) of stillbirths undergoing autopsy with placental examination.

Microarray

Ninety-seven (74%) of the cohort had tissue samples sent for microarray testing. The annual breakdown of testing is summarized in Table 1. Eighty-seven (88.5%) of those sent were deemed to have sufficient quality of DNA to test (see Figure 2 for schematic). Sixty-six (75.6%) samples yielded results. Eleven (16.7%) of the successful arrays yielded abnormal results (details summarized in Table 2). Two of these abnormal results were thought likely to be responsible for fetal demise.

Failed Microarrays

The most likely cause of failure was thought to be poor DNA quality caused by delay between fetal demise and testing. Maceration was noted in all 21 failure reports: 17

Table 1. Genetics results in study stillbirth population detailed by year.

	2011	2012	2013	2014	2015	Total
All stillbirths	69	72	45	57	59	302
SB postmortems (%)	30 (43.5)	39 (54.2)	31 (68.9)	21 (36.8)	37 (62.7)	158 (52.3)
Nondysmorphic at PM (%)	26 (81.2)	35 (89.7)	25 (80.6)	17 (80.9)	28 (75.7)	131 (82.9)
Microarrays performed	5	27	23	13	19	87
Normal arrays	2	19	15	7	12	55
Abnormal arrays	1	4	0	4	2	11
Failed array (%)	2	3	8	2	5	21 (23)
Requests for QF-PCR	1	9	13	7	8	38
Normal QF-PCR	1	8	9	3	7	28
Abnormal QF-PCR	0	0	0	0	0	0
Failed QF-PCR (%)	0	1	4	4	1	10 (26.3)
Total microarray and QF-PCR	6	36	36	20	27	125
Total abnormal results (percentage of requests)	1 (16.7)	4 (11)	0 (0)	4 (20)	2 (7.4)	11 (8.5)

QF-PCR, quantitative fluorescent polymerase chain reaction.

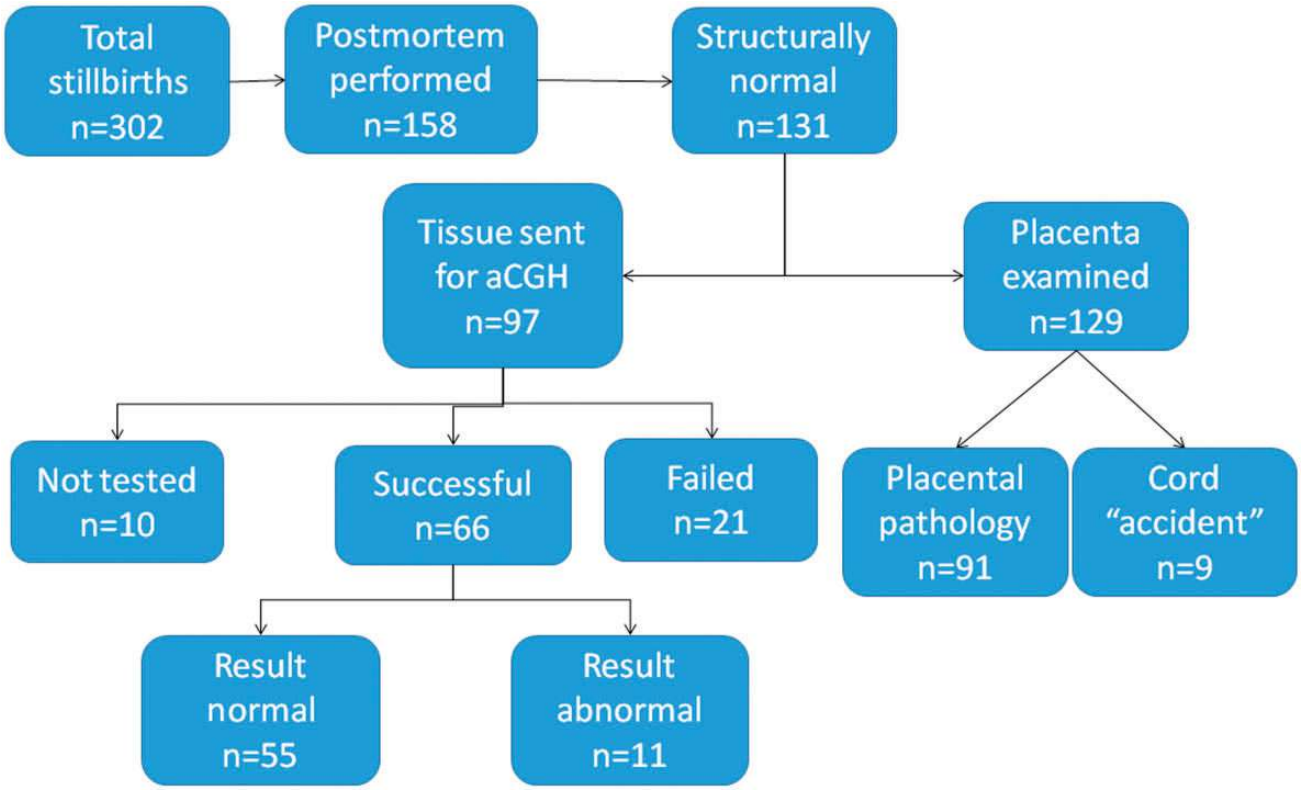


Figure 2. Comparison of genetic testing and placental pathology within the cohort study.

severe, 3 moderate, and 1 mild. Eleven of the failed array samples had successful testing for aneuploidies with QF-PCR all of which were normal, as per workup flowchart (see Figure 3).

Further Analysis

The IUGR population within the cohort could be considered at a higher risk of genetic mutation. We calculated individualized growth centiles as per the methods

Table 2. Details of genetic abnormalities identified on microarray analysis.

Proband	Array Abnormality	PM Findings	MDT Impression	Genomic Coordinates
I Term male	1.6 Mb del Ch12 between p12.2 and p12.1	Placenta: vasculitis, funisitis, and chorioamnionitis Neuropath: hypoxic ischemic insult	Incidental (Maternally inherited)	arr[GRCh37] 12p12.2p12.1 (20,870,874-22,482,438)x1 mat
II Term male	1.1 Mb del Ch15 Between q13.1 and q13.2	Bulky placenta Significant placental changes suggestive of Impaired glucose Tolerance or raised BMI	Incidental (Maternally inherited)	arr[GRCh37] 15q13.1q13.2 (29,213,945-30,351,627)x1 mat
III Term Male	1.63 Mb duplication of 16p13.11	Placenta: meconium staining with a cord of excessive length containing a true knot Neuropath: hypoxic ischemic stress	Unlikely to contribute to Stillbirth Paternal sample not available, implications unclear for family	arr[GRCh37] 16p13.11 (14,893,698-16,521,281)x3
IV 25/40 Female	6.34 Mb deletion on Ch14 between q21.1 and q21.2	Placental ischemia and infarction >80%	Incidental (maternally inherited)	arr[GRCh37] 14q21.1q21.2 (39,521,150-45,860,099)x1 mat
V 27/40 Male	Incomplete Trisomy 4 (likely mosaic)	External only Placenta showing fibro-obliterative changes	Likely causative No parental DNA	arr[GRCh37] 4p16.3q35.2 (106,643-190,717,003)x3
VI Term Female	A 52.66-Mb deletion of Xp22.33p11.22	Internal: horseshoe kidney Placenta: fetal thrombotic vasculopathy, villitis of unknown etiology Neuropath: hypoxic neuronal damage and reactive gliosis	Likely causative Associated with Turner phenotype No parental DNA	arr[GRCh37] Xp22.33p11.22 (168,551-52,832,021)x1
VII Term Female	0.29MB deletion of Ch2 at position p16.3	Nil External only Placenta showed massive Perivillous fibrin deposition	Incidental but significant for family *NRXN1* gene (nonlethal) Maternally inherited	arr[GRCh37] 2p16.3 (51,120,467-51,408,871)x1 mat
VIII Term Male	1.6 Mb duplication of Xp22.31	External only Placenta showed multiple areas of infarction and hemorrhagic disruption of the plate	Unlikely to contribute to stillbirth. Familial implication unclear No parental DNA	arr[GRCh37] Xp22.31 (6,457,080-8,138,497)x2
IX Term Male	1.4 Mb and 0.76 Mb deletions of 22q11.21	Placenta: hypocoiled cord, immature villi and changes of hemodynamic compromise Neuropath: early hypoxic damage	Unlikely to contribute to stillbirth. Familial implication uncertain No parental DNA	arr[GRCh37] 22q11.21(18,898,672-20,301,472)x1, 22q11.21(20,719,544-21,480,262)x1
X Term Female	1.6 Mb deletion of Xp22.31	Placenta—multiple foci of infarction accounting for >50% by volume	Unlikely to contribute to stillbirth. Familial implications uncertain. No paternal DNA	arr[GRCh37] Xp22.31 (6,470,571-89,111,237)x1
XI 36/40 Male	3p26.3 duplication	Placenta—unremarkable, widespread, neurological gliosis	Incidental/unlikely clinical significance as no OMIM morbid genes in region	arr[GRCh37] 3p26.3 (383,060-2,614,394)x3

BMI, body mass index; MDT, Multidisciplinary Team Meeting; OMIM, Online Mendelian Inheritance in Man; PM, Post mortem.

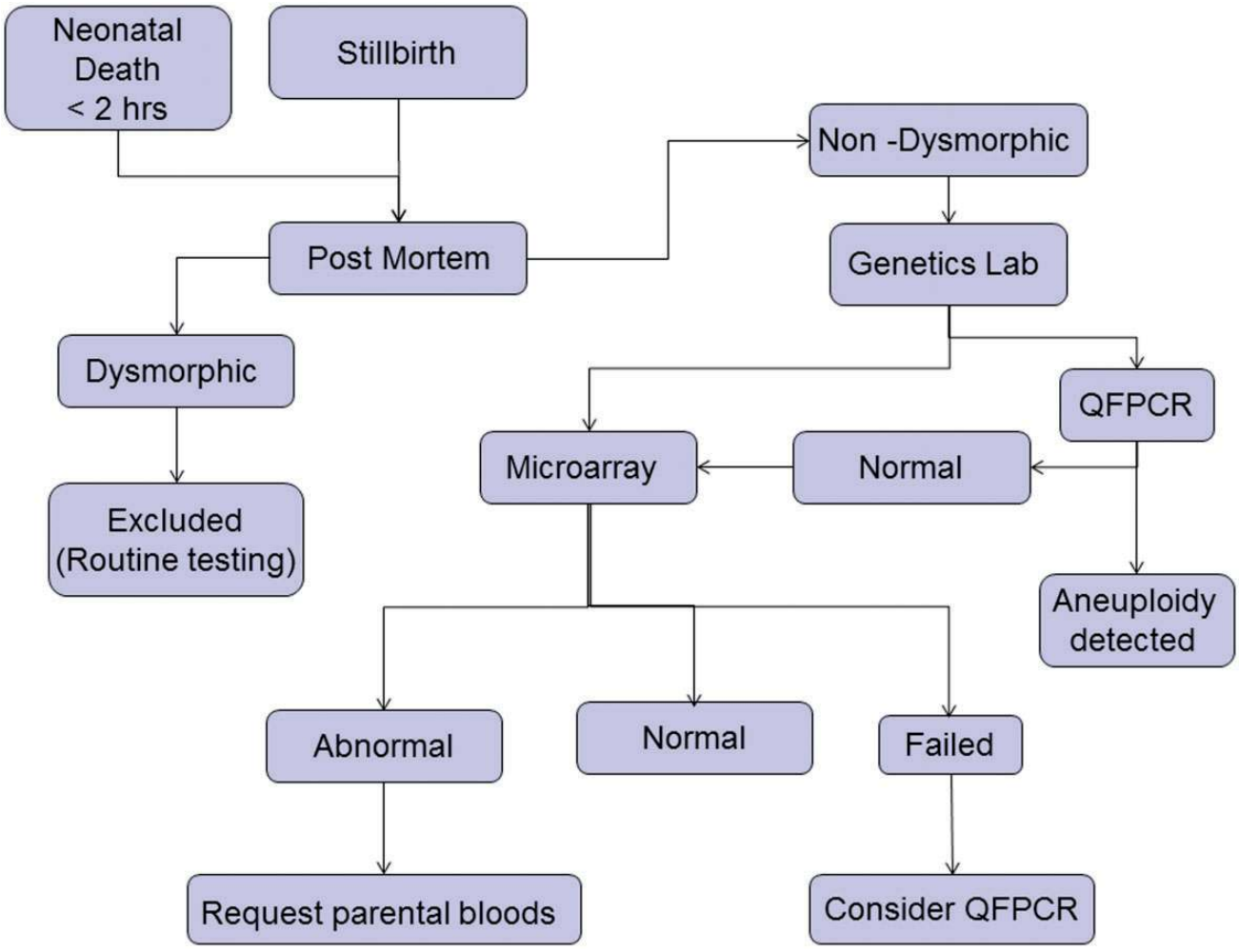


Figure 3. Flowchart describing the traditional course of genetic testing for a stillborn fetus within the study center.

ofde Jong et al.[22] IUGR was then defined by a centile score of less than 10. The number of cases was insufficient to warrant statistical consideration, and thus, these results were cross-checked with the normal and abnormal microarray results in a scatter graph to illustrate any underlying pattern. It was not apparent from our cohort that any subgroup was particularly at the risk of genetic abnormalities (Figure 4). Further, we analyzed results regarding gestational age at delivery, as chromosomal anomalies are thought to be overrepresented in early loss. However, we saw no relationship between CNV and gestational age or birth weight at delivery.

Discussion

The past few years have seen a general decrease in the overall number of stillbirths, particularly in developed countries.[23] This reduction has been largely due to improvements in obstetric care and better fetal monitoring. In view of this, the proportion of deaths attributable to genetic cases may be expected to rise thus supporting an increased role for genetic testing in the future.[24] Genetic testing may allow us to gain greater understanding of the mechanisms underlying stillbirth and thus drive the stillbirth rate down further. There have been studies of the utility of microarray analysis in pregnancy loss and stillbirth[7,9] showing improved sensitivity for genetic anomaly detection in comparison to traditional karyotyping. This increased sensitivity is also bolstered by improved successful test rate when the DNA quality is compromised.[25] There has been a large study by the Stillbirth Collaborative Research Network[26] looking at the diagnostic utility of different investigations for stillbirth in general. This study showed that the most informative investigations were placental pathology and fetal autopsy; however, genetic testing also showed usefulness especially when associated with congenital anomalies. In 11.9% of these cases, a cause was attributed to conditions suggested by genetic results. This is clearly encouraging, but expectations must be tempered in the study of non-dysmorphic stillbirths. Our study highlights diminished returns (a causative result found in 3%) from genetic

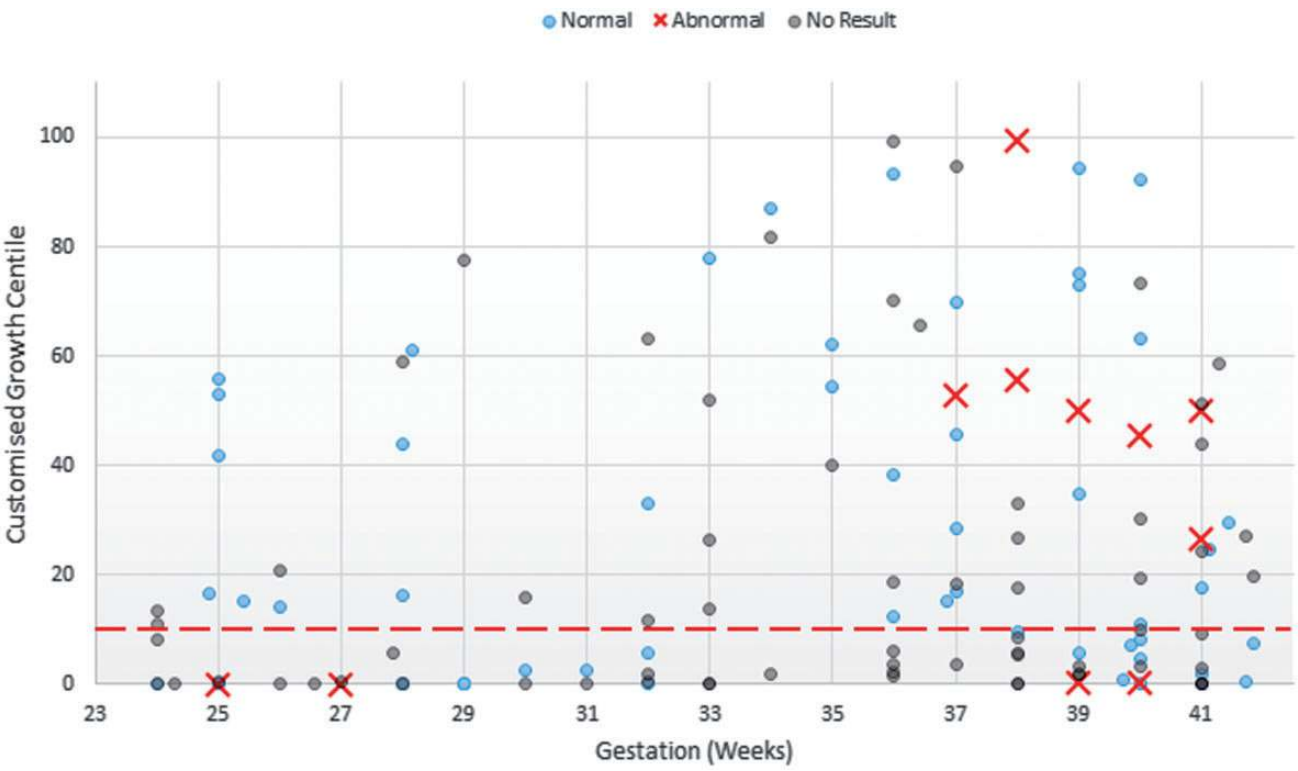
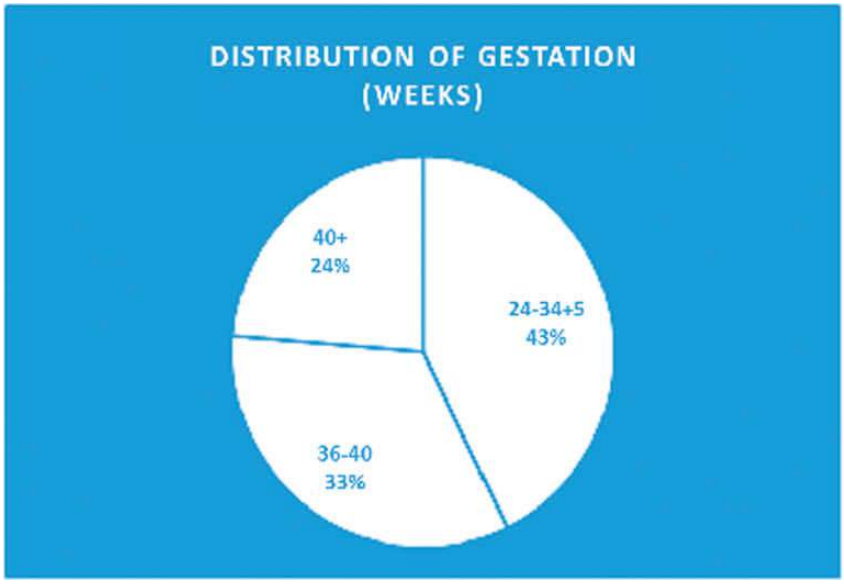


Figure 4. Microarray results relative to their personalized growth centiles and their gestation. The broken line marks definition of IUGR (below the 10th centile).



Male	54
Female	77
Ratio (M:F)	0.7:1
IUGR	66
Non IUGR	65

Figure 5. Left—Cohort as defined by gestational milestones. Right—Summary of sex and body habitus within the cohort.

screening in the absence of dysmorphism or congenital anomalies and suggests a considered approach to testing guided by experienced assessment.

In a qualitative study of parents affected by stillbirth, the most common reason for parents to authorize a fetal postmortem is to find a cause for their baby's death.[27] In this study, 21% of parents who were dissatisfied with their decisions at the time of consent for postmortem wished that more investigations had been performed. This insight supports consideration of genetic testing as

an adjunct to postmortem examination including minimally invasive postmortem which is growing in popularity.[28] However, is genetic testing truly of value in all cases? Recent studies have shown that genetic abnormalities may occur with greater frequency in the stillbirth population, compared to live births, most often associated with dysmorphism or structural abnormality,[29] but this study considers the nondysmorphic stillbirth population.

From 131 nondysmorphic stillbirths with no structural abnormalities, 97 underwent genetic testing revealing 11 cases of stillbirth with an anomaly detectable by microarray analysis. Of these cases, 2 were felt likely to account for the stillbirth: incomplete trisomy 4 and deletion of the small arm of chromosome X in a female. The clinical impact of placental and umbilical cord pathology was greater, accounting for the death in a total of 79 cases (61%) with a further 21 cases showing pathological changes thought to be contributory to the demise of the fetus. Excluding the cord pathology, about which little can be done, changes that would affect subsequent management were identified in 89 (69%) cases. In an era of economic restraint, it is important to ensure that testing is valid and relevant. To decrease the number of stillbirth, we need to hold systemic reviews where we look at events surrounding the stillbirth and identify markers of risk, such as altered fetal movements, reduced growth velocity, or IUGR. Customized charts may allow us to better predict those babies where growth is not meeting the genetic potential, but microarray is unlikely to answer why. Consent for genetic testing is important and nuanced. Not only can there be clear positive and negative results but uncertain results and unsolicited results as well. One such unsolicited result was present in a case with the deletion of the *NEUREXIN* gene in a fetus. Although this was not thought contributory to the stillbirth, it did correlate with an undisclosed family history of mild intellectual impairment. Thankfully this incidental result was well received by the family although it highlights the risks when testing at genomic level. Placental pathology can be interpreted independently informing subsequent management without active parental engagement at a difficult time. Discussion of the placental pathology alongside abnormal clinical findings, as part of significant adverse event review, may further enhance our understanding of contributing factors and identify markers for future screening.

Intrauterine Growth Restriction

IUGR is a significant cause of perinatal morbidity and mortality. There is increased recognition that IUGR should be recognized as a diagnostic cause of stillbirth rather than an association, and this diagnosis would reduce the incidence of stillbirth of unknown etiology. Most recent definitions use software to define a fetus growth potential based upon sex and constitutional characteristics at the beginning of pregnancy. This process allows for a customized growth centile calculation based on gestation and weight compared to the optimum predicted.[15] The use of individualized charts increased the specificity of screening for IUGR and the adverse outcomes associated with growth below the 10th centile.[30] This correlates with our cohort where half of the infants can be classified as IUGR. We recognize that there are compounding factors in the calculation of weight in the stillborn infant with a need to reassess the gestation with reference to foot length and to adjust the weight if maceration is severe.[31] Further, if there was a delay in autopsy, we used the birth weight which was considered to be a more accurate. There are a range of etiologies for IUGR,[32] and its role in stillbirth is incompletely understood. The prevalence in our cohort would support ongoing research into this aspect of stillbirth.

Summary

The health system benefits from identifying the cause of stillbirth as monitoring can be appropriately targeted in subsequent pregnancies.[33] Pregnancy history, family history, photography, radiology, external examination, internal examination (including neuropathology), histology, biochemistry, microbiology, and genetic analysis are all used to varying degrees investigating fetal death. Overarching the utility of these tools is consent to investigate. While it is important to recognize the invaluable contribution which can be made to the understanding of stillbirth by examination of the placenta, clinic-pathological correlation may be compromised if there is no detailed autopsy. Genetic testing offers another minimally invasive tool to use in this investigation, but its employment needs to be under the careful consideration of those skilled in analysis of phenotype correlation with genotype. With this safeguard, the most appropriate genetic test can be requested including specific mutation analysis. Standard protocols for postmortem assessment of stillborn infants have been suggested for many years,[34,35] and genetic testing needs to be considered as a component within such protocols. Genetic studies may be indicated for the minority of nondysmorphic cases where placental pathology fails to identify a cause of stillbirth. It is important that parental samples are available for trio analysis and that parents are appropriately counseled regarding the risk of unexpected and uninterpretable findings so that informed consent can be taken.

Limitations

Our study was a retrospective review, and data collection was limited to postmortem reports and genetic results. As

with all stillbirth research, our data set was defined by our population and the selection bias inherent to postmortem consent.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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