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Statistical Approach to Prenatal Zygosity Assessment Following a Decade of Molecular Aneuploidy Screening

Sílvia Pires,[1] António J. A. Nogueira,[2] Odília Pinho,[1] Tiago Delgado,[1] Mário Sousa,[3] Rosário Santos[1] and Paula Jorge[1*]

[1] *Centre for Medical Genetics Doutor Jacinto Magalhães, National Health Institute Doutor Ricardo Jorge, Porto, Portugal*
[2] *CESAM & Department of Biology, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal*
[3] *Department of Microscopy, Laboratory of Cell Biology, Biomedical Research Multidisciplinary Unit, Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto, Portugal*

In twin pregnancy studies, molecular genetic techniques have rarely been used to determine zygosity, despite their known precision and accuracy. The present work aimed to assess the power of discrimination in zygosity assessment, using a set of microsatellite markers that were routinely used for aneuploidy screening by multiplex-PCR in a prenatal context. Rapid aneuploidy screening using a group of 20 microsatellite markers (STRs) located on chromosomes 13, 18, 21 and X has been performed in our lab for over 10 years, with a total of approximately 1,500 samples studied to date. A retrospective analysis of the 257 prenatal samples from multiple pregnancies was carried out. A subset of 14 cases presenting theoretical monozygosity were re-evaluated by the use of biostatistics tools accessed via the ZygProb website. Further monozygosity determination relative to dizygosity was calculated, given an estimated overall error value of 0.093%. The results show that monozygosity had been correctly determined in all our previously studied twins. This work demonstrates that accurate zygosity assessment can be achieved with the same STRs applied in aneuploidy screening with a high power of discrimination and a matching probability of over 99.999999%.

■ **Keywords:** twins, prenatal diagnosis, zygosity determination, genetic counselling, ZygProb

Twins of spontaneously conceived pregnancies occur on average at 13 per 1,000 maternities, one-third of which are monozygotic (MZ) (Hoekstra et al., 2008). In Portugal, the twinning among total pregnancy increased from 0.82% (1253/151 634) to 1.33% (1356/100 140) over the last 25 years; concomitantly, singleton pregnancies decreased from 150,361 to 100,026 (data publically available in source: Instituto Nacional de Estatística; INE — Portugal, http://www.ine.pt). This rising rate, similar all over the world, is attributed to both increasing reliance on infertility treatment modalities and delayed childbearing (Antsaklis & Partsinevelos, 2008; Guilherme et al., 2008).

One of the most critical aspects for the successful managing of twin pregnancies is the early detection of chorionicity, as some authors consider that the combined risk of fetal chromosomal abnormality and advanced maternal age is higher in dizygotic twins than in singleton gestations (Odibo et al., 2003; Sin & Tan, 2009). On the other hand, several particular complications, such as twin-to-twin transfusion syndrome (TTTS) seem to occur at a significant rate in monozygotic twin pregnancies (Suetersa & Oepkes, 2005; Tong et al., 2004). Chorionicity, which refers to the type of placentation, can be determined routinely by ultrasound during the first trimester of pregnancy; however, in the second trimester the determination of chorionicity becomes more complex (Antsaklis & Partsinevelos, 2008; Tong et al., 2004). At this stage, DNA studies can be used to precisely determine zygosity

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ADDRESS FOR CORRESPONDENCE: Paula Jorge, Centre for Medical Genetics Doutor Jacinto Magalhães, National Health Institute Doutor Ricardo Jorge, Molecular Genetics Unit (UMO-P), Praça Pedro Nunes, 88, 4099-028 Porto, Portugal. E-mail: paula.jorge@insa.min-saude.pt

and implicitly also dichorionicity (dizygotic fetuses are always dichorionic). These results have a significant impact in same-sex twins or in pregnancies without previous knowledge of chorionicity. DNA studies in the prenatal determination of zygosity are useful for the clinical follow-up during pregnancy, antepartum management and prognosis particularly in multifetal pregnancies (Guilherme et al., 2008). Information of zygosity can also be valuable in compatibility studies for organ or bone marrow transplantation, simply for the interest of parents or for future post-natal research; studies on twin cohorts have long attracted scientists in the medical genetics field (Painter et al., 2010).

Invasive prenatal diagnosis is offered routinely at our centre to pregnant women who have been identified as having an increased risk for a fetal genetic condition. Molecular screening of the most frequent chromosomal abnormalities is based on the analysis of markers using multiplex fluorescent-primed PCR (also known as QF-PCR), which involves the simultaneous amplification of short tandem repeats (STRs, highly polymorphic stretches of repetitive DNA sequences) located on the chromosomes of interest (13, 18, 21 and X; Cirigliano et al., 2004; Mann et al., 2004; Pertl et al., 1997). Using this method, results can be obtained within a few hours following fetal sampling, with the advantages of reducing parental anxiety, accelerating therapeutic measures in cases where abnormalities are identified, or assisting decision-making about selective reduction in multiple pregnancies. The robustness and reproducibility of the method provide a definitive result in 100% of the cases; additionally, the low cost and automation allow high throughput for large-scale analysis (Brown et al., 2006). In the particular case of multifetal pregnancies, the genotype of each twin is generated concurrently with the aneuploidy screening. The aim of the present work was the retrospective review of twin pregnancies that had been subjected to invasive prenatal diagnosis (PND) and QF-PCR for aneuploidy screening, with further statistical analysis of results so as to validate a panel of markers in the context of zygosity determination. Usually concordance at all loci is accepted as evidence of monozygosity (Sacchetti et al., 1999). Theoretically, however, a pair of twins can be concordant in all studied alleles simply per chance and still be dizygotic (DZ), hence the importance of applying a probabilistic calculus in the determination of zygosity (Erickson, 2007). In the present work a subgroup of 14 pairs was chosen that evidenced the same genotype across all studied loci. Biostatistics methods accessed via the ZygProb home page (http://genepi.qimr.edu.au/general/daleN/ZygProb; Nyholt, 2006) were applied in order to establish the exact probability for monozygosity in each pair of twins, following estimation of allele frequencies in our population and estimation of the inherent technical error rate. This study has led to the implementation of a statistical component in the assessment of zygosity in a prenatal context, using a non-commercial panel of unlinked markers.

Materials and Methods

Samples

Since 1999, 128 cases of multiple pregnancies were studied and now reviewed. With the exception of one triplet all other were duplets. The fetal samples (n = 257) were mostly amniotic fluid (volume 1–3.5 ml; mean 2.2 ml), but 3 were fetal blood (two obtained by cordocentesis and one through cardiocentesis) and one was cultured amniocytes. Additionally, maternal genomic DNA (gDNA) was obtained from two blood spots (Ø 4 mm) collected on Whatman 903 filter paper (Whatman, Kent, UK). Whenever possible, blood samples were also collected from the father. The marker profiles of the Portuguese population (100 alleles) were determined using gDNA from healthy individuals (unrelated accompanying healthy adults from both sexes that came to our centre and volunteered for this study). Studies were performed on gDNA obtained using ReadyAmp Genomic DNA Purification System (Promega, Madison, USA), according to the manufacturer's instructions.

Multiplex-PCR

Amplification of several highly polymorphic STRs, located on chromosomes 13, 18, 21 and X, was done by polymerase chain reaction (PCR) using Multiplex Master Mix (Qiagen, Hilden, Germany) in a final volume of 20μL. The 22 sets of fluorescently labeled primers were organized in four different PCR mixtures. Mix I: D13S634 [Hex], D13S742 [Hex], D13S1265 [6-Fam], D18S59 [Hex] and D21S1914 [6-Fam]; Mix II: D13S305 [Hex], D18S978 [Ned], D21S1411 [Hex], D21S1412 [6-Fam] and XHPRT [6-Fam]; Mix III: DXS1224 [Vic], DXS7593 [Vic], DXS8019 [Ned], DXS8067 [Ned], DXS8009 [Vic] and DXS8088 [Ned]; and Mix IV: D13S153 [6-Fam], D18S535 [Hex], D18S1371 [6-Fam] and D21S1414 [6-Fam] (in square brackets is the fluorescent dye employed in each primer pair). Incorporated in Mix III were specific primers for regions in genes *AMELX*, *AMELY* [6-Fam] and *SRY* [6-Fam]. Primer sequences were retrieved from the Genome Data Base (http://www.gdb.org), with exception of markers in Mix III that correspond to the Linkage Mapping Set panel 83 (Applied Biosystems, Foster City, USA). After amplification, 1 μl of multiplex PCR product was added in a mix containing 14 μl of deionized formamide and GeneScan-500 [Rox] size standard. Fragments were denatured for 5 minutes and immediately placed on ice for at least 2 minutes, resolved on the 3130*xl* Genetic Analyser, and analyzed using the GeneMapper® Software v4.0 (Applied Biosystems).

Statistical Analysis

Zygosity odds were established using ECLIPSE2 software obtained at http://genepi.qimr.edu.au/general/daleN/

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ZygProb (Nyholt, 2006). Reproducibility of amplicon size was carried out at the beginning of the experiment using 5 sample-replicates for each primer pair on a single DNA sample. The coefficient of variation (CV) was calculated for each pair of alleles. The maximum CV was used to set the overall error value to be applied in the odds determination. The log file generated with ECLIPSE2 was used to calculate the probability of monozygosity considering general principles. The pedfile used in ECLIPSE2 had to include information on both parents and offspring. All possible combinations between parents and fetuses (six combinations) were calculated and the output file provided the likelihood associated with 8 different settings: Full Siblings or Dizygotic Twins (full=DZ), Half Siblings, Unrelated (unrel), Monozygotic Twins (MZ), Parent-Offspring, Grandparent-Grandchild, Avuncular, First Cousins. Since the purpose of this work was to assess the probability of monozygotic twins against dizygotic twins, only the likelihood ratios (LR) for each of these two conditions were considered. The difference of likelihoods (LL – diff) between monozygotic and dizygotic twins could be used to compute the probability of two twins being monozygotic [P(MZ/DZ)], considering that:

$$LL - diff = \log[LR(MZ/DZ)] = Likelihood(MZ) - Likelihood(DZ)$$

$$LR(MZ/DZ) = 10^{\mathrm{LL-diff}}$$

$$P(MZ/DZ) = \frac{LR(MZ/DZ)}{1 + LR(MZ/DZ)}$$

However, genetic similarities between parents might act as a confounding factor (Choueiri et al., 2006). Thus, a similar probability was also computed for parents and the probability of twins being monozygotic was corrected accordingly. The corrected probability, $P_c(MZ/DZ)$, was computed by multiplying the probability of two twins being monozygotic, $P_t(MZ/DZ)$, by the probability of the parents sharing significant genetic information [i.e., unrelated through to identical], $P_p(unrel/MZ)$:

$$P_p(unrel/MZ) = \frac{LR_p(unrel/MZ)}{1 + LR_p(unrel/MZ)}$$

Probability of parents being 'related'.

$$P_1(MZ/DZ) = \frac{LR_t(MZ/DZ)}{1 + LR_t(MZ/DZ)}$$

Probability of monozygotic twins.

$$P_c(MZ/DZ) = P_t(MZ/DZ) \times P_p(unrel/MZ)$$

Corrected probability of monozygotic twins.

Results

Multiple Pregnancies

The majority of multiple pregnancies (n = 128) were conceived naturally, while in-vitro fertilization (IVF) occurred in three cases, one of which was from oocyte donation. Invasive PND was performed at mean 16.6 weeks gestation.

TABLE 1

Summary of the Results Obtained with the Panel of 20 STR Markers

Marker	Type of repeat	No. of alleles	Chromosome location	cM	Allele size range[a] (bp)	Observed heterozygosity	PIC[b]
D13S742	AC	18	13q12.12	24,2	339–375	0.910	0.903
D13S305	CTTT	13	13q13.3	35,9	411–451	0.836	0.816
D13S153	CA	15	13q14.2	47,8	203–233	0.867	0.855
D13S634	GAAA	15	13q21.33	67,5	456–492	0.873	0.861
D13S1265	AC	13	13q33.3	108,1	278–304	0.808	0.790
D18S59	AC	9	18p11.32	0,6	156–174	0.825	0.803
D18S535	GATA	8	18q12.3	36,4	128–158	0.736	0.697
D18S978	ACTC	5	18q12.3	36,6	239–255	0.705	0.651
D18S1371	TCTA	7	18q22.3	71,2	134–160	0.663	0.606
D21S1414	TATC	10	21q21.1	19,5	339–363	0.854	0.837
D21S1914	GT	10	21q21.2	24,5	202–220	0.830	0.810
D21S1412	TCTT	13	21q22.2	39,7	381–421	0.857	0.840
D21S1411	GATA	20	21q22.3	43,0	261–315	0.895	0.887
DXS1224	TG	6	Xp22.2	13,2	160–176	0.446	0.418
DXS8019	GT	10	Xp22.13	17,7	156–174	0.856	0.840
DXS7593	GT	8	Xp22.11	22,3	215–231	0.729	0.692
DXS8088	GT	6	Xq23	113,3	262–272	0.664	0.597
DXS8067	AC	7	Xq24	119,2	91–109	0.684	0.631
DXS8009	GT	8	Xq25	126,0	254–270	0.721	0.687
XHPRT	CTAT	8	Xq26.2	133,4	269–297	0.806	0.779

Note: cM — centimorgan; [a]obtained on a 3130xl Genetic Analyser using POP7, dye set filter D and a 36cm long capillary; [b]PIC — assessed according to Shete et al., 2000.

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Risk factors that called for the invasive PND were advanced maternal age in 80% of cases and increased nuchal translucency in 1.6% of the cases. Other less frequent referrals included ultrasound abnormalities, family history of hemophilia A, maternal cytomegalovirus seroconversion during pregnancy and a previous son with trissomy 21. Chorionicity determination by ultrasound analysis, at time of sample collection, was possible in 64.1% of the multiple pregnancies (82/128). This examination revealed a diamniotic, dichorionic (DADC) pregnancy in 73.2% (60/82) of the twins, a diamniotic with a monochorionic placentation (DAMC) in 22.0% (18/82), and a monoamniotic, monochorionic (MAMC) pregnancy in 3.7% (3/82). The triplet pregnancy representing 1.2% (1/82) is one of the three IVF cases and showed a triamniotic, trichorionic (TATC) placentation with one female and two male fetuses.

STRs: Analysis of 100 Alleles

For each of the polymorphic markers employed in this study, on 20 unlinked loci, values of heterozygosity and polymorphism information content (PIC; Shete et al., 2000) for the control population were determined based on the analysis of 100 alleles (Table 1). With the exception of DXS1224, all the markers showed a heterozygosity level greater than 66.3% (mean 77.8%). Although its level of informativity was relatively low (44.6%), it was decided to maintain this marker in the present study as it is part of a commercially available set of primers. The markers were combined in four different PCR mixtures according to the size-range of the amplified products and the fluorescent label (see materials and methods). Primers for sequences within the genes *AMELX*, *AMELY* and *SRY* were also incorporated in Mix III in order to assist in gender-determination and to enable the identification of X-chromosome abnormalities.

Twin Pregnancies: Aneuploidy Screening and Zygosity Determination

Aneuploidy screening was not possible in 1.2% (3/257) of the samples due to extreme maternal contamination of fetal samples, and consequently zygosity was not determined for the respective three twin pregnancies. For all fetuses the result of aneuploidy screening by QF-PCR was concordant with their karyotype. All, except one case of trissomy 21, had a normal number of chromosomes 13, 18, 21 and X. Overall zygosity assessment was possible in 125 multiple pregnancies (250 samples). Dizygotic pairs (DZ) occurred in 65.6% (82/125), presumed monozygotic (MZ) in 36.0% (45/125) of the twin pregnancies, and 0.8% (1/125) corresponded to the trizygotic pregnancy. Sex distribution was similar in the set of MZ twins (23 male : 22 female) and slightly increased towards male twins in the group of the DZ pairs (47 male : 35 female). The results consistent with MZ represented 31.2% (30/82) of the chorionicity-known pregnancies (n = 82), and of these, approximately one-third showed different placentas (n = 11). In contrast, like-sex twin pregnancies within the group of unknown chorionicity (n = 46), represented 60.9% (28/46) of the twins. Monochorionicity exclusion by QF-PCR was decisive in 46.4% (13/28) of those cases, allowing a standard follow-up of the pregnant women.

Statistical Assessment of Zygosity

Classification as DZ was attributed to opposite-sex or same-sex twins who differed in at least two microsatellite DNA markers. Monozygosity was presumed when same-sex twins shared the same genotype in all amplified loci, with a minimum of five informative markers. However, this minimum was empirically established and no probabilistic basis was used for the final result. Therefore, we used ZygProb excel sheets to test for the exact (using allele frequency) and approximate (using locus heterozygosity) random match probability of a DZ twin pair sharing both alleles at all markers, and resulting probability of correct zygosity assessment, for this combination of STR markers (Table 2). As seen in Table 2, the odds of a DZ pair being identical by descent at all alleles is 1 in $3E^{-9}$, at all alleles, using the 20 markers; accordingly, the probability of MZ/DZ is equal or superior to $2{,}89E^{8}$. These results indicate that our set of 20 STR markers has a power of exclusion of at least 0.9999999965.

Additionally, we also calculated our own inherent technical error rate (Table 3) and used the maximum CV obtained (0.093%) to calculate the average probability of MZ/DZ likelihood ratio (LR) in a subset of 14 cases of presumed monozygotic twins (Table 4). The corrected probability P_c(MZ/DZ), was used to test if similarities in twin genotypes are real or influenced by a genetic proximity between parents (probability obtained relative to the parents of pair 1 is an exception as the results are due to low number of amplified markers). A confirmed DZ twin pair was used as control.

Discussion

The observed increase in the twinning rate, as well as the ratio of dizygotic to monozygotic (DZ/MZ) pregnancies, is similar to that previously described for various populations (Derom et al., 2001; Hall, 2003). Recent studies directly correlate assisted reproduction technology to the monozygotic twining increase rate (Corsello & Piro,

TABLE 2

Exact and Approximate Random Match Probabilities for the STRs Used

	Exact probability	Approximate probability
Probability of a DZ pair sharing both alleles at all markers	0.000000003	0.000000003
Average certainty of twin pair being MZ (%)	100.000000	99.99999965
Odds for MZ compared to DZ	295759900.0	289391752.9

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TABLE 3

Inherent Technical Error Rate: Variability (Degree of Uncertainty) Associated with Each Marker Determination

Marker	Allele 1 (%)	Allele 2 (%)	Average CV (%)	Maximum CV (%)
D13S742	0.144	0.142	0.143	0.144
D13S305	0.073	0.056	0.065	0.073
D13S153	0.069	0.073	0.071	0.073
D13S634	0.032	0.040	0.036	0.040
D13S1265	0.400	0.111	0.256	0.400
D18S59	0.090	0.092	0.091	0.092
D18S535	0.068	0.091	0.080	0.091
D18S978	0.089	0.083	0.086	0.089
D18S1371	0.067	0.104	0.085	0.104
D21S1414	0.019	0.006	0.012	0.019
D21S1914	0.079	0.072	0.076	0.079
D21S1412	0.066	0.066	0.066	0.066
D21S1411	0.075	0.084	0.079	0.084
DXS1224	0.047	0.046	0.046	0.047
DXS8019	0.074	0.074	0.074	0.074
DXS7593	0.078	0.098	0.088	0.098
DXS8088	0.096	0.096	0.096	0.096
DXS8067	0.108	0.121	0.114	0.121
DXS8009	0.099	0.161	0.130	0.161
XHPRT	0.079	0.073	0.076	0.079
Average	0.093%	0.084%	0.088%	0.093%

2010). Our small number of IVF-conceived twins does not allow any further reflection. In the present study no significant correlation was found between chorionicity or zygosity and the sex of the fetuses, although we have observed an increase in the number of male as opposed to female fetuses in the dizygotic group of the naturally conceived pregnancies. Determination of chorionicity in multiple pregnancies can be of great significance in the field of prenatal diagnosis and genetic counselling, particularly in instances where clinical management can be influenced (Carroll et al., 2005; Guilherme et al., 2009). Moreover, prenatal diagnosis of zygosity is important for assessment of risk when one fetus is known to be affected by a specific disorder, to determine genetic risk for a fetus whose twin is affected with a genetic condition for which a specific genetic test is not available, management of fetal death/reduction, and to evaluate the risk of structural anomalies, especially in like-sex twins where diagnosis of chorionicity remains undetermined (Chen et al., 2000; Derom et al., 2001).

This report describes the evaluation of the power of discrimination of a subset of polymorphic markers applied to zygosity determination in a prenatal context following aneuploidy screening. Commercially available kits have also been evaluated for zygosity determination (Guilherme et al., 2008; von Wurmb-Schwark et al., 2004; Yang et al., 2006). There was no marked difference in zygosity assessment between our panel and the multiplex-PCR kits described in the literature, all with a high power of discrimination. However, when compared to identification-kits, our methodology, besides fast and sensitive, is also cost-effective as it only requires additional analysis of data already generated from the aneuploidy screen (QF-PCR). Besides this, it also allows more reliable exclusion of maternal contamination, as compared to single nucleotide polymorphism (SNP) analysis (Hannelius et al., 2007) or routine multiplex ligation-dependent probe amplification (MLPA) studies (Gerdes et al., 2005; van Opstal et al., 2009), highlighting its significant advantage in a prenatal context, particularly if QF-PCR becomes widespread as the method of choice in future prenatal diagnostic procedures (Cirigliano et al., 2009).

Although the average confidence level for a correct designation of a twin pair sharing all alleles for the 20 markers, as MZ, is greater than 99%, in practice it is not uncommon to have inconclusive allele determination. Unlike others (van Opstal et al., 2009), our inconclusive results were not due to maternal cell contamination (MCC) because fetal allele profile was always compared to the mother, and when severe MCC was observed no further analysis was performed. Rather, our main reasons (Table 4) were attributed to neomutation, the technical phenomenon of allele dropout or artefacts resulting from critically small amounts of sample.

The probabilistic approach to zygosity diagnosis proved to be extremely useful as these 20 STR markers presented a power of exclusion of at least 0.99999965 between duplets in our cohort. These results are of particular interest in

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TABLE 4

Likelihood Ratios and Probabilities Associated with 14 Pairs of Presumed Monozygotic Twins and Their Parents (Error Value 0.001)

Twins identification	No. of amplified STR_t	LR_t(MZ/DZ)	LR_p(unrel/MZ)	P_t(MZ/DZ)(%)	P_p(unrel/MZ)(%)	P_c(MZ/DZ)(%)
Pair 1	7	13.5884	0.533	93.145	34.781	32.397
Pair 2	7	68.517	$8.04E^{16}$	98.562	100.000	98.562
Pair 3	11	54.988	$2.08E^{10}$	98.214	100.000	98.214
Pair 4	16	832.722	$2.79E^{24}$	99.880	100.000	99.880
Pair 5	11	178.607	$7.61E^{26}$	99.443	100.000	99.443
Pair 6	14	350.341	$1.00E^{25}$	99.715	100.000	99.715
Pair 7	11	240.315	$1.00E^{25}$	99.586	100.000	99.586
Pair 8	10	269.525	$3.87E^{19}$	99.630	100.000	99.630
Pair 9	12	374.914	$2.73E^{13}$	99.734	100.000	99.734
Pair 10	17	846.480	$1.76E^{26}$	99.880	100.000	99.880
Pair 11	15	653.094	$1.61E^{26}$	99.847	100.000	99.847
Pair 12	17	780.400	$1.35E^{34}$	99.872	100.000	99.872
Pair 13	17	834.842	$1.35E^{34}$	99.880	100.000	99.880
Pair 14	17	690.459	$2.23E^{20}$	99.854	100.000	99.854
DZ control	16	$3.286E^{-12}$	$2.55E^{37}$	0.000	100.000	0.000

Note: t – monozygotic twins; p – 'unrelated' parents; c – corrected probability of monozygotic twins.

clinical practice, with particular emphasis in like-sex pairs where exact knowledge of the chorionicity is unknown, because they allow us to calculate the likelihood ratio of monozygosity simultaneously with common aneuploidy screening even in critically small samples, besides retrospective guarantee of sample collection in both sacs (Winsor et al., 2010). On the other hand, prenatal zygosity assessment is useful in instances where chorionicity remains undetermined and its knowledge is likely to influence clinical management; for example, in the case of multiple pregnancies complicated by the intrauterine demise of one of the twins, in cases of early onset discordant fetal growth, or prior to selective reduction in multifetal gestations. Equally important is the knowledge of zygosity for perinatal follow-up as monozygosity, as well as dizygosity after IVF, are associated with adverse outcomes (Källén et al., 2010); and better counselling to parents regarding their individually unique, twin offspring. Furthermore, the results obtained may assist other researchers planning to use a similar strategy, by helping to choose the best combination of markers and by establishing the respective probability of correct zygosity assignment.

Familial MZ seems more common than suggested in the literature, but underlying causes are still unclear. An important way to understand those mechanisms can be the study of twinning following natural and medically assisted conceptions. Furthermore, the tendency to conceive dizygotic twins appears to be influenced not only by genetics but also by environmental issues, and the rise in the DZ twinning rate with maternal age seems independent of genetics effects. Investigation of the particular case of MZ pregnancies to unravel those apparent divergences is currently being undertaken in our department.

In conclusion, STR analysis is a reliable tool for the prenatal determination of the zygosity independently of the chorionicity and the fetal sex, using a multiplex-PCR for 20 polymorphic markers followed by probability/statistical analysis.

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