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Antibiotic Prophylaxis before second-trimester Genetic Amniocentesis (APGA): a single-centre open randomised controlled trial

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Objective To compare procedure-related pregnancy loss after second-trimester genetic amniocentesis in women given an antibiotic prophylaxis and controls.

Methods Prospective, open randomised controlled single-centre study between January 1999 and December 2005 at Artemisia Fetal Maternal Medical Centre. A follow-up within 4 weeks after the procedure was done.

Of 36 347 eligible women, 1424 refused to participate and 34 923 were enrolled and randomised with unequal chance of selection, 21 991 were assigned to treatment group and 12 932 were assigned to the control group, and did not receive any placebo. Oral azithromycin, 500 mg per day, was administered 3 days before amniocentesis. The primary endpoint was the procedure-related pregnancy loss. The secondary endpoint was the rate of preterm premature rupture of membranes.

Results The rate of abortion related to the amniocentesis was 7/21 219 women (0.03%, 95% CI 0.009–0.057) in the intervention group, and 36/12 529 (0.28%, 0.28–0.30) in controls ($p = 0.0019$). The rate of preterm premature rupture of membranes was 14/21 219 (0.06%, 0.031–0.101) in the intervention group, and 140/12 529 (1.12%, 0.94–1.30) in the control group ($p = 0.001$).

Conclusions Antibiotic prophylaxis before second-trimester amniocentesis reduced the risk of abortion and of rupture of the membranes. Copyright © 2009 John Wiley & Sons, Ltd.

KEY WORDS: amniocentesis; antibiotic-prophylaxis; prenatal Cytogenetics; fetal and placental pathology; general Cytogenetics

INTRODUCTION

Amniocentesis was first introduced for foetal karyotyping in clinical practice in 1966 (Steele and Breg, 1966). The rate of foetal death after genetic amniocentesis is generally thought to be very low. In the past three decades, many studies and collaborative trials have reported that the rate of foetal deaths ranges from 0.06% to 2.9% (Table 1) (JAMA, 1976; Simpson *et al*., 1976; MRC Working Party on Amniocentesis, 1978; Bartsch *et al*., 1980; Crandall *et al*., 1980; Tabor *et al*., 1986; The Canadian Early and Mid-trimester Amniocentesis Trial (CEMAT) Group, 1998; Caughey *et al*., 2006; Eddleman *et al*., 2006). The rate established by the Centre for Disease Control and Prevention—0.5% lost pregnancies after amniocentesis (JAMA, 1976; Simpson *et al*., 1976)—is often cited; however, the source of this data has not been reported. The one randomised trial, which was done by Tabor and colleagues in 1986 (Tabor *et al*., 1986), reported a rate of 1.3%; however, it focused on low-risk patients. Most of the other investigations have been retrospective studies with insufficient clinical evidence.

Although information about the likelihood of foetal death after genetic amniocentesis could help pregnant women to decide whether or not to undergo this invasive test, it might never be established definitively, given the ethical and organisational issues for any randomised controlled trial (Eddleman *et al*., 2006). Moreover, the inherent risk of pregnancy loss between 16 and 24 weeks of gestation can complicate identification of any deaths that might be caused by amniocentesis (Seeds, 2004).

The difference between reported rates of foetal loss could stem from the range of factors that might predispose women to amniocentesis-induced pregnancy loss. However, only a few of these, such as maternal age, the skill of the operator, bleeding during the pregnancy, and a history of second-trimester miscarriage, have been assessed as possible determinants of foetal death (Papantoniou *et al*., 2001). We need to understand not only how these factors act, but how we might counteract them.

In normal circumstances, the amniotic cavity is regarded as a sterile environment, due partly to the interaction of the cervical epithelium, placental membranes, and cellular components of the placenta (Talmi

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Table 1—Studies on rate of foetal deaths after midtrimester amniocentesis

Study (year)	Number of participants	Foetal deaths	Control type
JAMA (1976)	995	2.9%	Matched
Simpson *et al*. (1976)	965	1.0%	Unmatched
MRC Working Party on Amniocentesis (1978)	2428	2.4%	Matched
Crandall *et al*. (1980)	2000	1.3%	Unmatched
Bartsch *et al*. (1980)	1000	1.3%	None
Tabor *et al*. (1986)	2242	1.3%	Randomised
The Canadian Early and Mid-trimester Amniocentesis Trial (CEMAT) Group (1998)	2090	1.9%	None
Eddleman *et al*. (2006)	3096	0.06%	Unmatched
Caughey *et al*. (2006)	30 893	0.83%	None

et al., 1991; Romero *et al*., 1993; Svinarich *et al*., 1997). However, microbial agents have been shown to cross the membranes (Galask *et al*., 1984), even when the membranes are physically intact. This suggests that bacterial proliferation within the amniotic cavity could be prevented by factors such as the presence of antimicrobial peptides, and that disruption of this steady state could result in infection, and its ensuing consequences (Espinoza *et al*., 2003).

Infections following amniocentesis have so far been analysed as consequences of the procedure; few studies have yet analysed whether these infections can cause other adverse outcomes (Ayadi *et al*., 1998; Wu *et al*., 2007). Pregnancies which are accompanied by subclinical infections in the decidua, the chorion, or the amniotic compartment generally have a high risk of complications (Romero *et al*., 1998; Goldenberg *et al*., 2000). Chronic inflammation or infections tend to increase the production of hormones and cytokines in the uterus and membranes, and can cause preterm premature rupture of membranes (Romero *et al*., 1998). Moreover, infectious agents are present in the amniotic fluid and membranes of asymptomatic women during amniocentesis (Bashiri *et al*., 1999). One study (Perni *et al*., 2004) detected *Mycoplasma hominis* or *Ureaplasma urealyticum* in more than 15% of samples of amniotic fluid at the second-trimester, and others have reported the presence of other bacteria (Bearfield *et al*., 2002).

We speculated that preterm premature rupture of membranes could be caused by reactivation of an infection that is latent in the membrane. If entry of the needle caused inflammatory trauma and detachment of a small portion of the membrane, it could trigger the latent infection to cause local inflammation by activation of the cytokine-prostaglandin cascade, contractions, and local oedema. This inflammation could result in preterm premature rupture of the amniotic sac, which, in turn, could either spontaneously heal or cause preterm labour or foetal death (Ayadi *et al*., 1998; Goldenberg *et al*., 2000; Wu *et al*., 2007).

Therefore, we aimed to assess whether prophylactic sterilisation of the membranes with an antibiotic 3 days before amniocentesis would reduce pregnancy complications after the procedure. A retrospective study of antibiotic prophylaxis, which had a small sample size, different antibiotics, and different operators, did not show efficacy (Gramellini *et al*., 2007). Our study was designed to compare the procedure-related pregnancy loss and of preterm premature ruptures of membranes in women given antibiotic prophylaxis and in controls after second-trimester genetic amniocentesis, within 4 weeks of the procedure.

METHODS

Study design and participants

We did a prospective randomised controlled trial, between January 1999 and December 2005, at the Artemisia Fetal–Maternal Medical Centre, in Rome. The prenatal diagnosis department at this centre is the largest in Italy (Dallapiccola *et al*., 1998), and since 2000 has done more than 7000 prenatal invasive procedures every year.

We enrolled all pregnant women who requested amniocentesis in the second-trimester, and who were at least 18 years of age. We excluded patients who (1) had a non-viable foetus (before or after randomisation), (2) had major foetal abnormalities (before or after the randomisation), (3) leakage of amniotic fluid, (4) bleeding in the past week, (5) fever, (6) use of any antibiotics within the past 14 days or of long-acting injectable penicillin at any time, (7) had a known allergy to the specific antibiotic used, or (8) were private patients of the operator or study collaborators. We obtained written consent from every patient, after giving them detailed information about the possible negative effects of the study. The study was approved by the Centre's ethics committee.

Procedures

Eligible women were randomly allocated to either the treatment or control group as soon as they consented to participate, and at least 15 days before the amniocentesis. To randomise participants, we used a customised randomisation programme that generated a random number for each participant, one after the other, with unequal ratio (2 : 1) in favour of odd number linked to the antibiotic group, with no restriction in the process. The program was provided by a computer engineer expert in clinical trials, separate from the study team, to exclude bias. Eligible women who were randomly allocated to the treatment group were given 500 mg oral azithromycin at 24-h intervals for 3 days before the amniocentesis procedure. Controls were not given any placebo.

Every woman underwent an ultrasound scan on the day of the amniocentesis. Gestational age was calculated from the last menstrual period and from this ultrasound examination. Only one operator with more than

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20 years' experience (C.G.) performed all amniocenteses and was the only participant of the study who was blinded of the treatment allocation of patients. He did the amniocentesis under continuous ultrasound guidance using a 21-gauge, 20-cm needle. Procedures were done 5 days every week, with between 20 and 35 procedures per day. The first 1 mL of amniotic fluid was discarded and a further 20 mL were withdrawn for cytogenetic analysis. Patients for whom needle insertion failed and was repeated (28 because of failure of the amniocyte culture, and four because of sampling failure) were included in the trial and were counted only once. In twin pregnancies, one needle-insertion was done in each sac. We did a further ultrasound scan, and then discharged women 30 min after the amniocentesis; no bed-rest was suggested.

All patients were asked to report any complications to the centre in the 4 weeks after the procedure. At 4 weeks, women were asked to visit the centre for a clinical and ultrasonographic examination; if unable to attend this follow-up visit, they were asked to undergo a scan elsewhere, and to provide the results to the study staff. The study staff who collected the follow-up were not blinded to treatment allocation. All women who did not return for their prescribed check-ups were contacted by telephone. Clinical and ultrasonographic examinations were done at the centre for 5920 (27.9%) of the treatment group and 3320 (26.5%) of controls, at other private offices for 9633 (45.4%) of the treatment group and 5801 (46.3%) of controls, and by the National Health Service ambulatory care for 5666 (26.7%) of the treatment group and 3408 (27.2%) of controls ($p > 0.05$ for all three comparisons). Moreover, all women who had any kind of problem (even anxiety) or possible complications were examined at our centre (ultrasound scan and swab). With this method we checked directly all the complications occurring in the study. In all normal cases the follow-up was performed by the referring private obstetricians, National Health Service ambulatory care, and our centre itself, and the pertinent information was collected directly from the patients or from the doctor mainly by phone.

Every 6 months, all procedures were checked for quality control and certified throughout the 7-year duration of the trial. An accredited certification body (CERMET) inspected the procedures according to ISO 9001 : 2000. The internal and external inspectors verified that all the phases outlined in the protocol were strictly complied to and that no manoeuvring of the database had taken place. Moreover, they controlled that the database was closed and blinded to all the participants in the study.

The primary endpoint was the procedure-related pregnancy loss within 4 weeks of the procedure. Foetal loss was defined as absence of a heartbeat on an ultrasound reading and absence of foetal activity.

The secondary endpoint was the rate of preterm premature rupture of membranes within 4 weeks of the procedure. Rupture of membranes was suspected if fluid leaked from the vagina, and was confirmed by ultrasound documentation of reduced volume of amniotic fluid (<5th percentile), direct visualisation of pooling in the posterior vaginal fornix and cervix, crystallisation of amniotic fluid (ferning), and a test with nitrazine, insulin-like growth factor binding protein-1, or both.

Statistical analysis

Prior to initiation of the trial, we enrolled 3000 women into a pilot trial (data not published); there was no foetal loss in the treatment group and a rate of spontaneous abortion of 0.2% among controls. We calculated that a sample size of 9631 women in each group would be required to demonstrate a reduction in rate of foetal loss from 0.2% among controls to 0.015% in the antibiotic group (power = 90%, alpha = 0.05 two-sided). The latter percentage was derived by an arbitrary estimate of a rate of foetal loss with antibiotic prophylaxis (Johnson *et al.*, 1996; Wilson *et al.*, 1997; The Canadian Early and Mid-trimester Amniocentesis Trial (CEMAT) Group, 1998).

Our pre-established statistical plan stipulated that, if the pilot study had shown that the effects of the intervention were negative ($p > 0.005$), we would stop the study. Conversely, if the pilot study showed a positive effect of the antibiotic ($p \leq 0.005$), we planned to increase the group receiving antibiotic prophylaxis in a ratio of 2 : 1 for ethical reasons. Analysis of the pilot study resulted in a χ^2 score of 7.128, with an associated p value of 0.0049 for the difference in primary outcome between the intervention and control arms. Therefore, on the advice of our panel of statisticians and the data safety and monitoring committee (DSMC), we changed the randomisation ratio from 1 : 1 to 2 : 1. For this ratio, and assuming a 3% loss to follow up, we calculated that we would need to enrol a minimum number of 19 262 women in the treatment group and a minimum of 9631 in the control group (at least 29 759 in total).

On the basis of this sample size and the number of amniocentesis procedures done every year by the operator (C.G.), we planned a 7-year study. No interim analysis was programmed.

Only procedure-related pregnancy loss or preterm premature rupture of membranes within 4 weeks of the amniocentesis procedure was counted as outcome events. The intention-to-treat analysis included all women who received amniocentesis and antibiotic treatment.

Because our study was a pragmatic clinical trial to identify the effectiveness of antibiotic prophylaxis for prevention of foetal loss and preterm premature rupture of membranes after amniocentesis, we excluded women who voluntarily terminated their pregnancies from our analyses. We compared outcome measures as proportions between groups with the χ^2 test. This trial was registered as ISRCTN30372886.

RESULTS

Figure 1 shows the trial profile. Of 36 347 eligible women, 1424 refused to participate, and thus 34 923 were enrolled and randomised. Of these, 34 466 women

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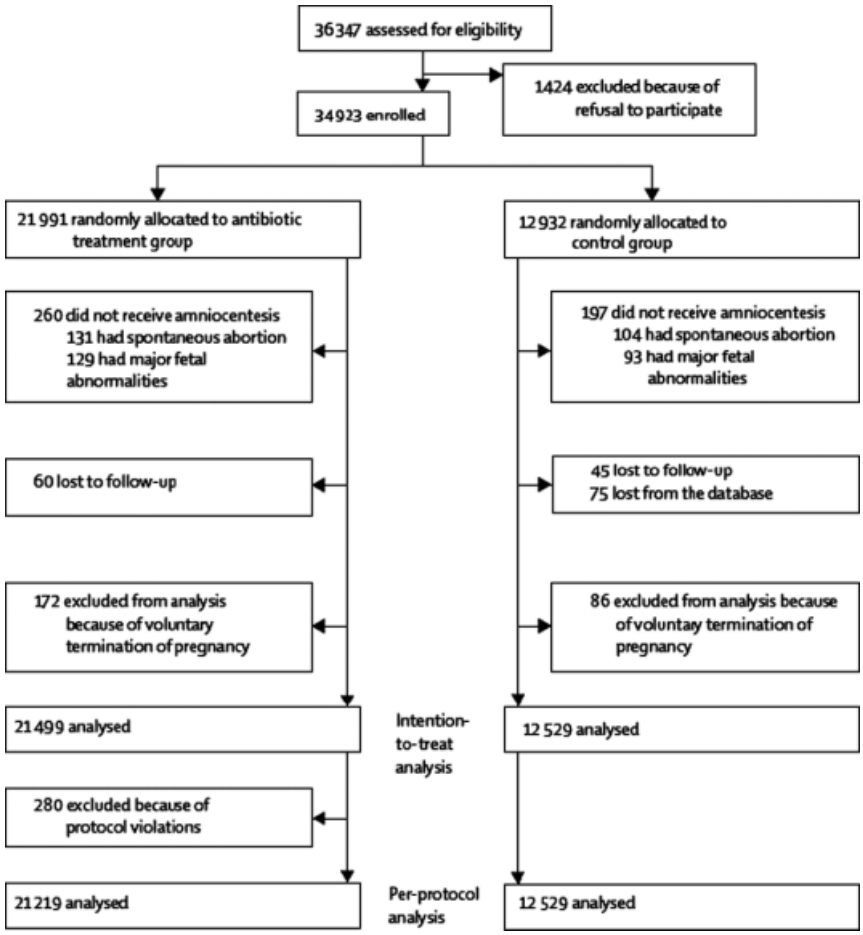

Figure 1—Trial profile

underwent amniocentesis; 457 randomised women were excluded from the study because they did not receive amniocentesis: 235 (0.66%) women had spontaneous abortions and 222 (0.63%) had major foetal abnormalities; 105 patients were lost to follow-up because we were unable to contact them. Corruption of computer files caused us to lose information for 75 women.

Complete information on pregnancy outcomes 4 weeks after the procedure was available for 34 286 (98.2%) women; 258 (0.75%) of them terminated the pregnancy after they received the karyotype results, and were excluded from all analyses; 34 028 women remained in the intention-to-treat population.

Of these 34 028 women, 33 748 (99.1%) completed the study as per protocol and their baseline characteristics are shown in table 2. The 280 protocol violations consisted of 48 (0.1%) patients who were given the wrong antibiotic, 100 (0.3%) who took the antibiotic at the wrong time, and 132 (0.4%) who interrupted the course of treatment before the three 500 mg doses had been taken because of side effects.

We confirmed foetal loss within 4 weeks of the procedure for seven of 21 219 women in the intervention group, at a rate of 0.03% (95% CI 0.009–0.057), and for 36 of 12 529 women in the control group (0.28%, 95% CI 0.28–0.3, $p < 0.0001$). Table 3 shows that 14

Table 2—Demographic and baseline characteristics

Variables	Antibiotic group ($n = 21219$)	Control group ($n = 12529$)
Mean age (years)	33.5 (3.89)	33.6 (3.89)
White	21 155 (99.7%)	12 593(99.8%)
Chronic hypertension	191(0.9%)	100 (0.8%)
Smoker during pregnancy	1803 (8.5%)	1127 (9.0%)
Primipara	9760 (46%)	5888 (47%)
Multipara	11 459(54%)	6641 (53%)
Gestational age, weeks	16.7 (1.035)	16.6 (1.035)
Twins	348 (1.64%)	214 (1.71%)
Procedures failures	21 (0.1%)	11(0.09%)
Double insertion	12 (0.06%)	5 (0.04%)
Indication for procedure		
Age ≥35	10 397 (49%)	6013 (48%)
Anxiety	7851 (37%)	4886 (39%)
Positive screening for foetal chromosomal abnormalities	1230 (5.8%)	664 (5.3%)
Family history of genetic disorder	190 (0.9%)	125 (1%)
Personal* history of risk	3076 (14.5%)	1866 (14.9%)

Data are median (IQR), number (%), or mean (SD).
* Ultrasound markers, infection, previous chromosomal abnormality, previous genetic disorders, assisted reproductive technologies, previous exposure to teratogens

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Table 3—Comparison of rates of foetal death and preterm premature rupture of the membranes (pPROM) after amniocentesis

	Treatment group	Control group	*p* value	Relative risk
Foetal deaths*	7 (0.03%, 0.009–0.057)	36 (0.28%, 0.28–0.3)	<0.001	0.11 [0.05–0.26]
pPROM*	14 (0.06%, 0.031–0.101)	140 (1.12%, 0.94–1.3)	<0.001	0.06 [0.03–0.1]
Foetal deaths after pPROM*	1 (7.1%)	20 (14.3%)	0.73	..
Foetal death†	7 (0.03%, 0.009–0.057)	36 (0.28%, 0.28–0.3)	<0.001	0.11 [0.05–0.26]
pPROM†	14 (0.06%, 0.031–0.101)	140 (1.12%, 0.94–1.3)	<0.001	0.06 [0.03–0.1]

Data are number (%), number (%, 95% CI), or RR (95% CI).
* Per-protocol analysis.
† Intention-to-treat analysis.

of 21 219 (0.06%) women [0.031–0.101] in the intervention group had preterm premature rupture of membranes within 4 weeks of the procedure, compared with 140 of 12 529 women (1.12%) [0.94–1.3] in the control group ($p < 0.0001$). The procedure-related pregnancy loss after preterm premature rupture of membranes did not differ between the two groups (Table 3). One of 14 (7.1%) women in the treatment group who experienced membrane rupture had a foetal death within 4 weeks of the procedure, compared with 20/140 (14.3%) in the control group ($p = 0.73$).

No serious side effects related to antibiotics were recorded during treatment. Because the participants were not masked to observers at week 4, adverse events and symptoms could be properly diagnosed and reported. Of 21 991 women, 126 (0.6%) had nausea, vomiting, or diarrhoea, and six (0.03%) had a moderate allergy which spontaneously resolved after the interruption of treatment. By contrast, none of the 12 529 controls had any such symptoms ($p < 0.0001$). In the control group, 256 (2.1%) women had morning sickness or nausea and vomiting of pregnancy, compared with 488 (2.3%) in the treatment group ($p > 0.05$).

DISCUSSION

Our results showed that a prophylactic treatment with azithromycin before second-trimester amniocentesis reduces the procedure-related pregnancy loss and preterm premature rupture of membranes, compared with controls. However, antibiotic treatment does not prevent foetal loss in women who have preterm premature rupture of membranes. Therefore, antibiotic prophylaxis seems to reduce foetal death by prevention of preterm premature rupture of membranes.

We chose azithromycin, a macrolide antibiotic, because it is safe in pregnancy, does not have teratogenic effects, and is effective against a wide range of microbes, including *Mycoplasma* (Sarkar *et al*., 2006). Moreover, azithromycin has a long estimated half-life (Ramsey *et al*., 2003), good tolerability, and few side effects (Johnson *et al*., 1996; Wilson *et al*., 1997). However, it is possible that the incidence of azithromycin-related side effects was over-estimated because questions about side effects were limited to the azithromycin-treated group (women in the control group were not asked about specific symptoms).

Macrolides might also protect the amniotic membrane by acting as generic peripheral immuno-modulatory agents (Tamaoki *et al*., 2004), and by promoting local nitric oxide activity to increase the local blood supply, and to antagonise oxidative stress within the cells (Ianaro *et al*., 2000). However, specific research on the role of membrane infections in preterm premature rupture of membranes could have helped in the choice of a specific antibiotic.

We set our primary outcome to within 4 weeks after the procedure on the basis of studies which reported that miscarriages happened at a median interval of 21.5 days after amniocentesis (Tabor *et al*., 1986); that foetal deaths were most common within the first 30 days after amniocentesis (Giorlandino *et al*., 1994); that rates of foetal death after 20 weeks of gestation were similar in women who had early amniocentesis and those who had midtrimester amniocentesis (Johnson *et al*., 1999); and that most foetal deaths (90.5%) occurred within 4 weeks of the procedure (Eddleman *et al*., 2006). We believed that a follow-up till birth was not suggested because it would have introduced a bias related to prognostic factors which differed from the short-term effect of amniocentesis. However, we agree that the study would have been more complete should we have also been able to follow-up the patients to delivery. This would have enabled us to evaluate whether the protection provided by the antibiotic lasted throughout the pregnancy.

For obvious organisational reasons it was not possible to administer placebo to the patients. In fact, the protocol contemplated that the prophylaxis be administered outside the prenatal diagnosis centre after randomisation, in the 3 days preceding the patient's visit to the centre.

We made the decision to continue the study for the entire 7 years without interim analyses in order to minimize the potential for bias, which can be engendered from knowledge of the interim results. Recent studies support such theories (U.S. Department of Health and Human Services, 2006). Moreover an interim analysis could have resulted in early interruption of the study, which would have been criticised as the results would have been viewed with scepticism (Montori *et al*., 2005). Finally only in the pilot study, the DSMC was interested in the surveillance and statistical analysis. The DSMC was not used in the antibiotic prophylaxis before second-trimester genetic amniocentesis (APGA) trial because of the absence of interim analyses.

The protocol predicted a period of 7 years to ensure that the minimum total number of 29 759 would be

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reached. When we did the statistical analysis, at the end of the planned study, we were quite surprised to have exceeded the minimum number required by 5164 women. Such an excess was the result of both the fact that we had underestimated the number of subjects who would have joined our trial as well as the fact that over the years the number of women being treated at the Centre increased.

Computer randomisation is generally accepted to be a gold standard since it is extremely risky to be in possession of a pre-constituted randomisation list, as it may allow knowledge, during or prior to the interview with the recruited patient, in which group the patient would be allocated. In our case it was essential to avoid such a bias. The authors chose the dynamic allocation of patients, one by one. With this kind of allocation, it was possible to better respect the second principle of randomisation, the principle of eliminating the selection bias between the groups as compared to the sub-experimental (recruitment), which may influence the randomisation process. In other words, this method is the better system to avoid that the referring health care provider is aware of the next allocation and may (even unknowingly) influence enrolment of participating subjects (Schulz, 1995; Schulz and Grimes, 2002)

No blocking was done in the randomisation process due to the possibility to insert a bias in the study. Infact, improved balance comes at the cost of reducing the unpredictability of the sequence. Although the order of interventions varies randomly within each block, a person running the trial could deduce some of the next treatment allocations if they discovered the block size (Schulz, 1995).

The authors were fully prepared and aware of the fact that at the end of the period determined in December 2005, an imbalance between the two groups could have appeared. In fact, at the end of the study there was a surprisingly high prevalence (3%) of the subjects treated with the antibiotic. This mistake occurred as a result of the use of a dynamic allocation and without an upper limit of participants randomisation programme. In any case, even today, an imbalance is considered to be unavoidable and could change continuously in the course of the randomisation. The mathematical rule states that the imbalance will move towards zero when the number of interrogations moves towards infinity.

The APGA trial was designed to eliminate all possible confounders and to concentrate the results exclusively on a unique variable—the antibiotic. For this very reason, it was imperative to eliminate any bias in this phase, and to administer the study in only one centre by only one operator. The strength of the trial in fact was that all possible confounders were eliminated, leaving only the antibiotic prophylaxis to 'make a difference' between the two groups tested. In particular, the same institute, the same operator, and no differences between demographic aspects in both groups are elements absolutely fundamental in re-assuring that the real discriminating element was the antibiotic. The criteria to define foetal loss or preterm premature rupture of membranes were based on very well-established objective and restricted criteria. Choice of other end-points more operator dependent would have introduced the possibility of bias, which would have affected the ability to verify the exclusive efficacy of the antibiotic. Finally, we used wide inclusion criteria, a short duration of treatment, and had good patient compliance because of the convenient dosing regime.

Regarding external validity, our results might not be generalisable to different races or to women in other geographic areas who might have different types of infections and baseline physical conditions. Ultimately, multicentric trials among centres of similar experience are needed to confirm the external validity of this study.

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Details of ethics approval

The procedures of the study received ethics approval from the institutional ethics committee responsible for human experimentation (PRSV-98-0012 - 12/09/1998).

Role of the funding source

The study sponsor, Italian Society of Prenatal Diagnosis and Fetal Maternal Medicine (S.I.Di.P.) had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had final responsibility for the decision to submit for publication.

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