The feasibility of early amniocentesis for fetal karyotyping

by

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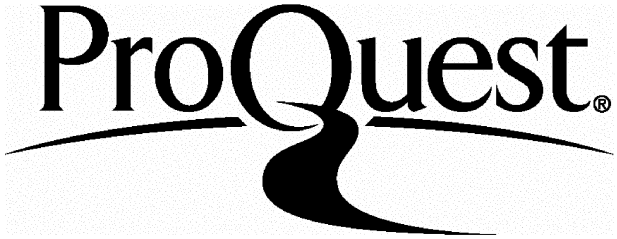

Abstract

This thesis investigates the feasibility of amniocentesis in the first trimester of human pregnancy for the diagnosis of fetal karyotype.

A cross sectional study of amniotic fluid from 125 pregnancies at 8-18 weeks' gestation, demonstrated an exponential increase in the total number of cells with gestation; however, the number of viable cells did not change significantly. A subsequent pilot study of amniocentesis in 56 women, prior to elective termination of pregnancy at 9-14 weeks' gestation, demonstrated culture success in all 39 cases where the fetal crown-rump length was >37mm, but in only 8 of the 17 (47%) where it was $\leq$37mm.

In a randomised study a sample was successfully obtained at the first attempt in 320 of 324 (98.8%) cases of early amniocentesis and in 323 of 326 (99%) cases of chorion villus sampling; culture and chromosomal analysis was successful in 98.1% and 99.4% of these cases respectively.

To limit the large proportion of amniotic fluid removed at early amniocentesis amnifiltration was developed. This is the filtration of amniotic fluid to trap free cells whilst the fluid is returned to the amniotic sac. A series of experiments involving 218 patients undergoing elective termination of pregnancy at 10-14 weeks' gestation established that (i), a cellulose acetate filter with 0.8 μm pore size was the most efficient filter tested (ii) 20 mls amniotic fluid must be filtered to achieve the same number

of clones in culture as early amniocentesis, (iii) filtered cells could be cultured and karyotyped in a similar time interval to early amniocentesis, (iv) amnifiltration was not associated with bacterial or viral contamination of the amniotic fluid and did not significantly change the urea, electrolytes, proteins and enzyme concentration .

The safety and diagnostic accuracy of early amniocentesis remains to be determined by a randomised trial, but preliminary outcome results demonstrate comparable rates of livebirths following early amniocentesis and chorion villus sampling (92% versus 93.8%). However, fetal losses were higher following early amniocentesis than after chorion villus sampling (5.2% versus 1.2%).

Table of contents

Page

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I am indebted to Kypros Nicolaides, who trained me in the techniques of ultrasound guided needle aspiration of chorionic villi and amniotic fluid, and under whose expert guidance I gained considerable experience in all aspects of Obstetric ultrasound. I am fortunate to have benefited from his wide experience, encouragement and advice. I am also grateful to Mrs Karen Marks who provided karyotype analysis of the samples I obtained and whose expertise was invaluable in the cytological aspects of this study.

I was responsible for counselling patients, performing the pre-procedure ultrasound assessment and carrying out the invasive test. I performed all the cytological examinations to determine the total and viable cell content of amniotic fluid in early pregnancy. I designed the amnifilter, carried out all the procedures and examined the resultant cell cultures. I collected, analysed and interpreted all the data on the studies of amnifiltration.

Publications arising from this thesis

Byrne D, Azar G and Nicolaides K. Why cell culture is successful after early amniocentesis. Fet Diagn Ther 1991;6:84-86.

Byrne D, Marks K, Azar G and Nicolaides K. Randomized study of early amniocentesis versus chorionic villus sampling: a technical and cytogenetic comparison of 650 patients. Ultrasound Obstet Gynecol 1991;1:235-240.

Byrne DL, Marks K, Braude PR and Nicolaides KH. Amnifiltration in the first trimester: feasibility, technical aspects and cytological outcome. Ultrasound Obstet Gynecol 1991;1:320-324.

Byrne DL, Marks K, Braude PR and Nicolaides KH. Amnifiltration in the first trimester; an alternative to early amniocentesis to limit amniotic fluid removal. Prenat Diagn 1992;12(suppl):S151.

Figure and Table legends

Page

Chapter 1

Introduction

1.1 Incidence and implications of chromosomal abnormalities

1.1.1 Incidence and patterns in prenatal and postnatal series
1.1.2 How chromosomal abnormalities arise
1.1.3 Features of common chromosomal abnormalities
1.1.4 Socioeconomic consequences

1.2 Screening for chromosomal abnormalities

1.2.1 Maternal age
1.2.2 Family history
1.2.3 Maternal serum biochemistry
1.2.4 Ultrasonography

1.3 Diagnostic techniques in the second and third trimesters

1.3.1 Amniocentesis
1.3.2 Cordocentesis and placental biopsy

1.4 Diagnostic techniques in the first trimester

1.4.1 Chorion villus sampling
1.4.2 Early amniocentesis

1.5 Aims of the thesis

Chapter 1 Introduction

At present, detection of chromosomal abnormalities constitutes the commonest indication for prenatal diagnosis. Over the last 20 years most developed countries have established programmes based on maternal age for screening and amniocentesis at 16 weeks' gestation, for diagnosis of fetal chromosomal abnormality. Results from this procedure are generally available by 20 weeks' gestation. Although in the mid 1980's the ability to sample chorionic villi introduced the prospect of fetal karyotyping in the first trimester of pregnancy, recent publications have raised concerns about the safety and diagnostic accuracy of this technique. This thesis examines the feasibility and methods of early amniotic fluid sampling as an alternative to chorion villus sampling for fetal karyotyping in the first trimester.

1.1 Incidence and implications of chromosomal abnormalities

1.1.1 Incidence and patterns in prenatal and postnatal series

Fetal cytogenetic abnormalities affect up to 7.5% of all pregnancies (Wramsby et al 1987). Up to 90% of affected fetuses are spontaneously aborted in early pregnancy, and 4% end in perinatal death. Despite this high fetal loss rate, approximately 1 in 160 livebirths have major chromosomal abnormalities (Tolmie 1989). In a combined survey of 68,159 livebirths, the incidence of chromosomal abnormalities was 0.65%, whereas in a combined series of 8,841 spontaneous abortions the incidence was 40.9% (Hsu 1986)

The incidence of fetal chromosomal abnormality increases with maternal age and is higher in the first trimester of pregnancy than in the second trimester or at birth (Table 1.1; Connor and Ferguson-Smith 1991).

Maternal age (years)	Incidence of trisomy 21 (Down's syndrome)		
	At birth	At 16 weeks Amniocentesis	At 10 weeks Chorion Villus Sampling
20	1/1500	1/1200*	1/750*
25	1/1350	1/1000*	1/675*
30	1/900	1/700*	1/450*
35	1/380	1/300	1/240
37	1/240	1/190	1/130
39	1/150	1/120	1/75
41	1/85	1/70	1/40
43	1/50	1/40	1/25
45	1/28	1/22	1/13

* Estimated value

Table 1.1 Incidence of trisomy 21 with maternal age at different gestations of pregnancy (Connor and Ferguson-Smith 1991).

The pattern of chromosomal abnormality changes with gestation. For example, trisomy 16 which inevitably results in spontaneous abortion, will be represented in chorion villus or amniotic fluid cultures but not at birth. The most likely outcome for triploidy and 45,X is also pregnancy loss and these abnormalities constitute a high proportion of abnormalities found in abortuses (Table 1.2). The common trisomies which present in liveborn infants are those of chromosomes 21, 18 and 13, presumably because they have a less disruptive effect on fetal development. Autosomal abnormalities produce more severe clinical problems in the newborn than sex chromosome aneuploidies, whilst autosomal imbalance is almost invariably

associated with mental retardation and dysmorphism. Table 1.2 compares the distribution of different abnormalities in livebirths and spontaneous abortions, and was adapted from the large reported series reviewed by Hsu (1986).

Karyotype	SA	LB
Autosomal trisomy	52.3	21.7
Monosomy X	19.1	1.4
Triploidy	16.2	0.0
Tetraploidy	5.5	0.0
Other	6.9	76.9

Table 1.2 Comparative frequency (%) of different chromosomal abnormalities in spontaneous abortions (SA) and livebirths (LB; adapted from Hsu 1986).

1.1.2 How chromosomal abnormalities arise

The mechanisms for development of chromosomal abnormality are diverse, the most frequent causes are illustrated below.

Non-disjunction

Non-disjunction is the failure of separation of a chromosome pair after metaphase, and results in one of the daughter cells containing both chromosomes and the other neither, causing trisomy in the former and monosomy in the latter. It may occur at gamete formation (figure 1.1), usually in the first (figure 1.2), but occasionally in the second meiotic

division (figure 1.3) or rarely, during mitosis. The majority of cases (80%) of non-disjunction occur during oocyte formation, and the frequency of this increases with advancing maternal age. Less frequently, it may occur during spermatocyte formation, but it is not related to paternal age.

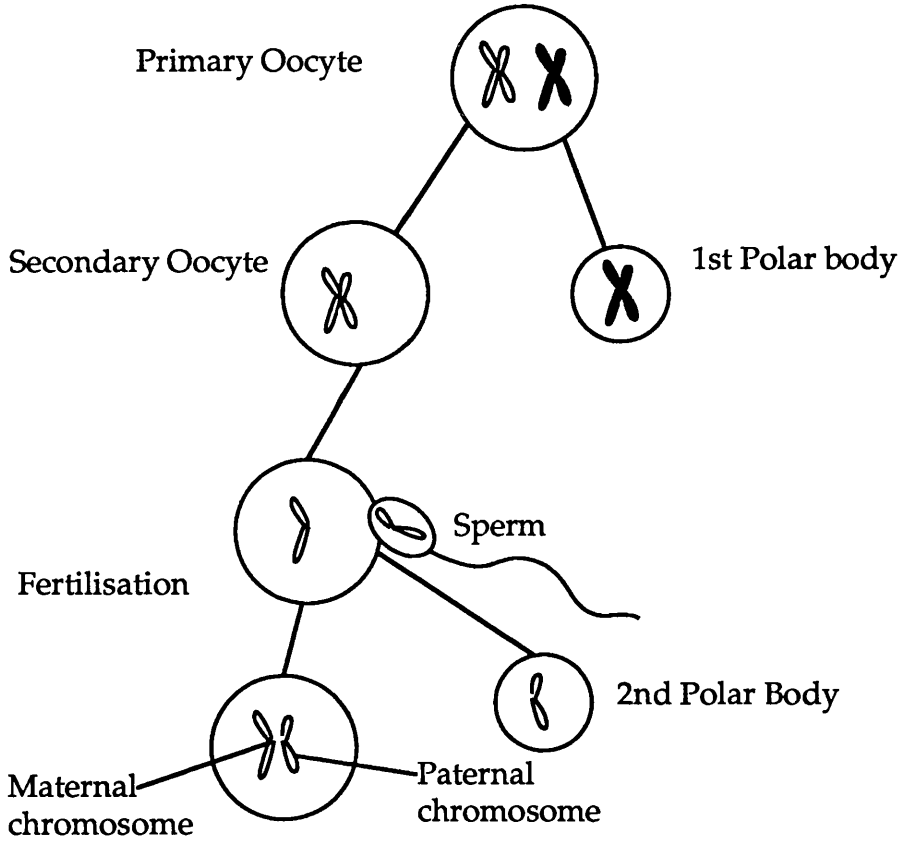

Figure 1.1 Normal meiosis resulting in a normal zygote

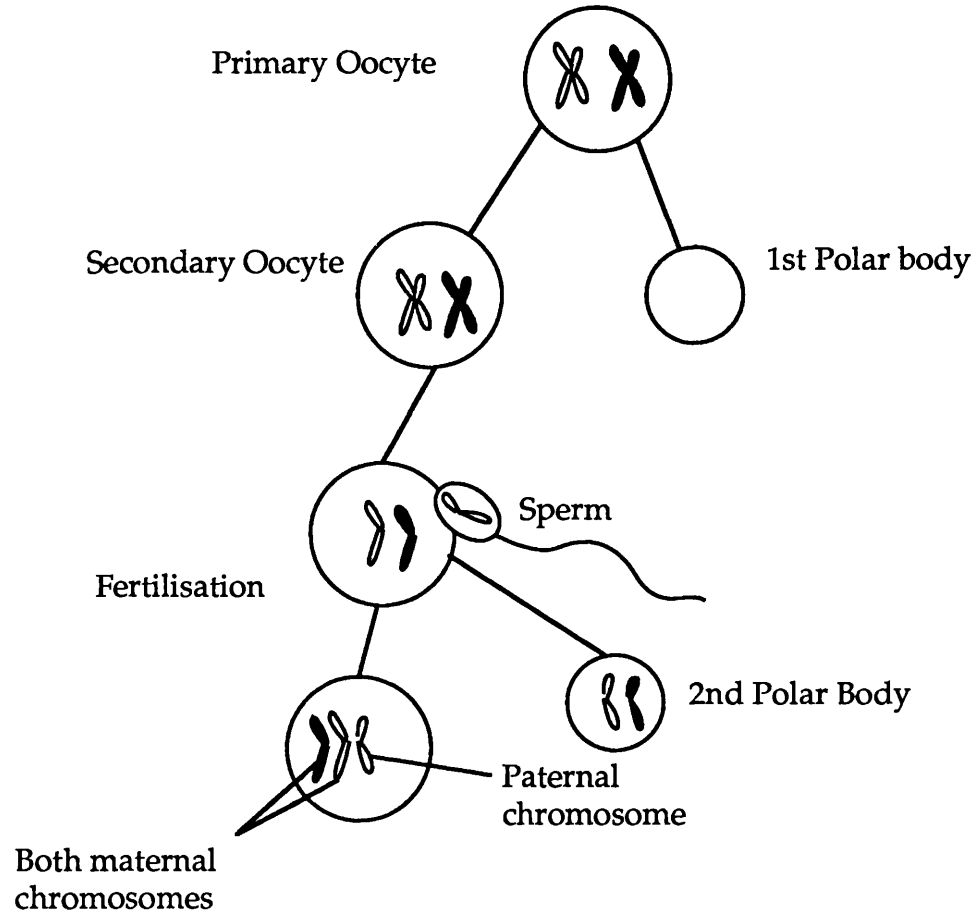

Figure 1.2 Non disjunction in the first meiotic division resulting in a trisomic zygote.

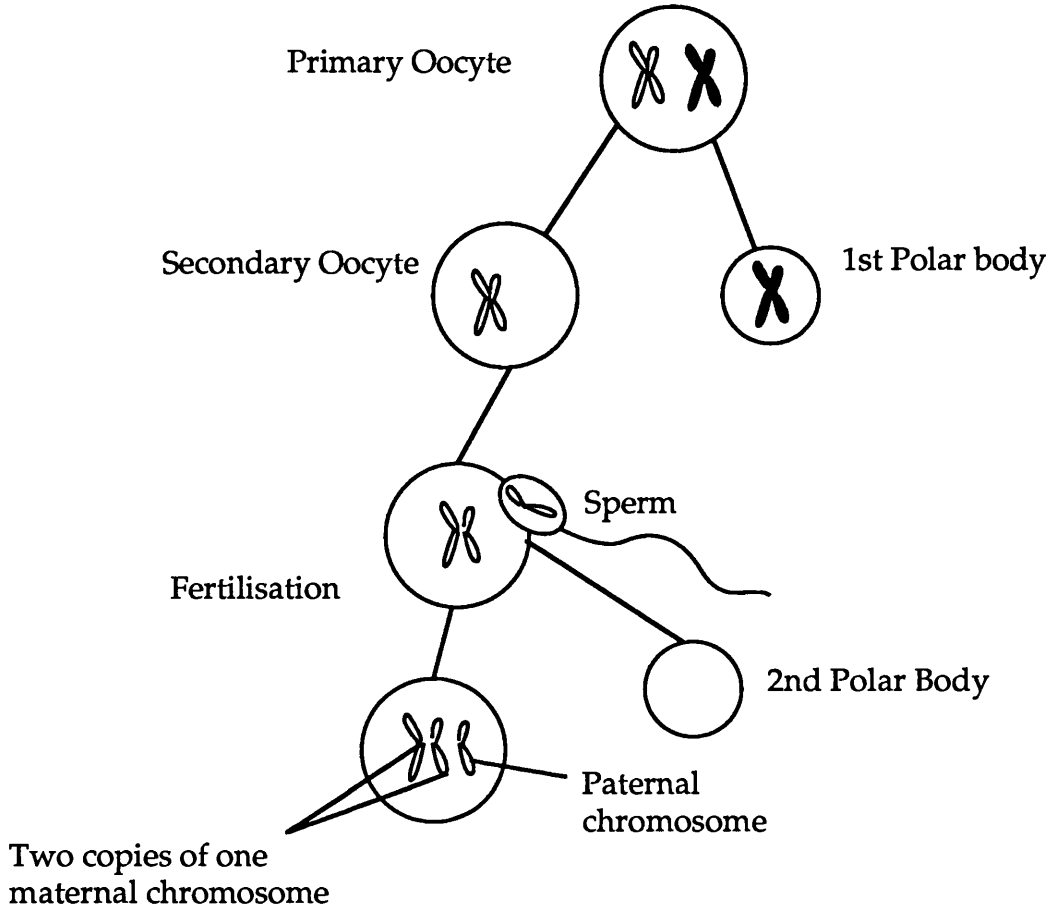

Figure 1.3 Non disjunction at the second meiotic division resulting in a trisomic zygote.

Anaphase lag

Anaphase lag is the delayed movement of a chromosome along the spindle at anaphase, such that it does not reach the pole of the cell before the nuclear membrane closes. This results in one of the daughter cells gaining an additional chromosome.

Reciprocal translocation

Reciprocal translocation is the exchange of genetic material from one chromosome to another. If the translocated segment is unimportant there will be no clinical consequences resulting from its repositioning. However, the individual will be a carrier of this balanced translocation, and may produce offspring with an unbalanced chromosomal complement. Translocations which cause breaks in critical segments or result in an unbalanced chromosomal complement will result in clinical consequences, usually mental handicap.

Centric fusion (Robertsonian translocation)

If two acrocentric chromosomes break at the centromere and join together, losing their short arms and centromeres, this is Robertsonian translocation or "Centric fusion" (Figure 2). Two such fused chromosomes are effectively carried as one and when inherited may represent an additional copy of one of the two fused chromosomes, resulting in trisomy. This most commonly involves chromosomes 13 and 14, and less frequently chromosome 21.

Mosaicism

In mosaicism the body contains two genetically different cell lines which have arisen from the same zygote. This results from either non-disjunction or anaphase lag in early embryo development and is distinct from chimerism, which is two cell lines arising from two different zygotes.

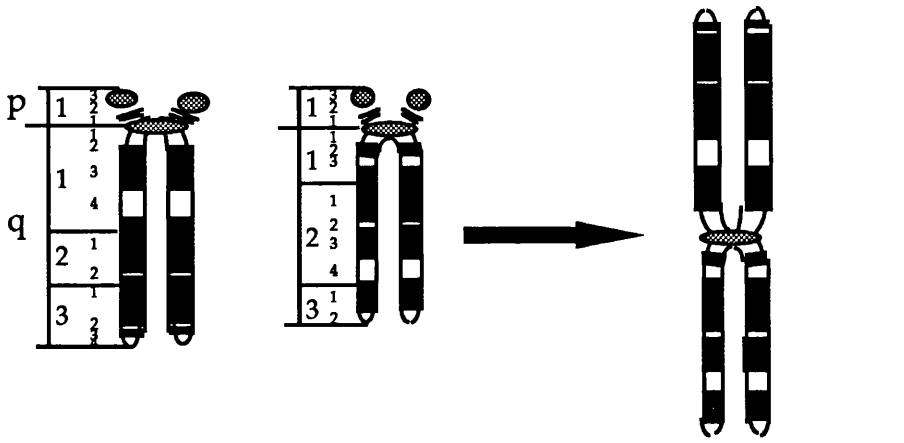

Figure 1.4 Robertsonian translocation involving chromosomes 13 and 14.

Partial deletions and duplications

Rarely small segments of chromosome are deleted, resulting in an unbalanced chromosomal complement. Examples include deletion of part of the short arm of chromosome 5 which results in Cri du Chat syndrome and mental retardation, or deletion in chromosome 13 which is associated with the congenital tumour, retinoblastoma.

Inversions

Inversions of chromosomal segments (two breakages and rotation of the segment through 180°, before rejoining) may include the centromere (pericentric inversion) or just one arm of the effected chromosome (paracentric inversion). If the involved segment is critical it will result in a clinical effect, if not the individual remains a carrier of the inversion but may produce chromosomal imbalance during subsequent meiosis, resulting in pregnancy loss or handicap in subsequent children. Certain inversions

are known to be associated with a high risk of producing unbalanced recombinants, such as inv(8)(p23q22), whereas others are at low risk of doing so, such as inv(1)(p13q21) (Kaiser 1988). Investigation of couples who experience recurrent miscarriages or have handicapped offspring may reveal such balanced rearrangements.

1.1.3 Features of common chromosomal abnormalities

This section provides details on the incidence and characteristics of the most common chromosomal abnormalities.

Trisomy 21 (Down's Syndrome, Mongolism)

The incidence of trisomy 21 or Down's syndrome (Down 1866) is 1 in 660 livebirths and it is the commonest cause of severe mental retardation (Penrose and Smith 1966). In the absence of prenatal diagnosis and therapeutic abortion over 800 affected children would be born annually in England and Wales (Cuckle and Wald 1990). In the majority of cases (95%) the parents have a normal genotype, but during gamete formation meiotic non-disjunction results in transfer of an extra chromosome to the recombinant (Scully 1978). The origin of the extra chromosome is maternal in about 80% of cases (Mikkelson 1982), possibly explaining the close relationship with maternal age. A minority (2%) of Trisomy 21 results from a de novo translocation (Valentine 1986). The clinical effect is indistinguishable from non-disjunction trisomy 21, but it is imperative that parents of such children are karyotyped to distinguish these cases from offspring of balanced translocation carriers where the recurrence risk is up to

100% (Tolmie 1989). If trisomy 21 originates from chromosomally normal parents the risk of recurrence is increased by about 0.3% above the maternal age-related risk (Cuckle and Wald 1990).

The majority of Down's syndrome children despite severe mental retardation (IQ: 25-50) learn to walk and talk and can integrate into society, with provision for their special needs. As adults they need constant supervision either within the family or an institution (Smith 1964, Valentine 1986). The characteristic facies of the Down's syndrome are well recognised (Hall 1966) as is the high association (30%) with congenital abnormalities like cardiac anomalies and duodenal atresia, which contribute to the high infant mortality rate (Hall 1966, Smith 1982).

Trisomy 18 (Edwards syndrome)

The incidence of Trisomy 18 is 1 per 3,000 livebirths, with a 3:1 female to male preponderance (Smith 1964), and its incidence is related to maternal age (Valentine 1986). It was first described by Edwards in 1960, when it was found to be due to trisomy of an E group chromosome. The cause is usually non disjunction during meiosis, rarely parental translocation and very rarely mosaicism, with a milder clinical effect (Smith 1982, Valentine 1986). During pregnancy the fetus may develop intrauterine growth retardation and at birth exhibits characteristic dysmorphic features including micrognathia, low set and sometimes malformed ears and clenched fists with overlapping fingers (Smith 1964 Valentine 1986). Cardiac and renal malformations are common (Warkany et al 1966) and death in the first year is the most frequent (90%) outcome (Smith 1964), those that survive show severe developmental delay (Weber 1967).

Trisomy 13 (Patau's syndrome)

Trisomy 13, found in 1 in 5,000 livebirths, was appreciated as a clinical entity as long ago as 1657 (Bartolinus 1657, Warburg and Mikkelson 1963), but not discovered to be due to trisomy until 1960 (Patau et al 1960). The majority of cases are due to parental non-disjunction, less are due to parental translocation and rarely mosaics (Valentine 1986). The characteristic dysmorphic features include midline facial cleft, hypotelorism, micropthalmia, holoprosencephaly, post axial polydactyly and rocker bottom feet (Patau 1960). Congenital heart disease is commonly associated (Warkany et al 1966) and 24% babies die in the neonatal period (Weber 1967), only 18% survive the first year, all of whom exhibit severe developmental delay (Smith 1982). Affected males are more likely to die in the first three months than affected females (Weber 1967).

47,XYY

This occurs in 1 in 1,000 males (Connor and Ferguson-Smith 1991) and was first described by Sandberg et al (1961). It is due to fertilisation by sperm with two Y chromosomes, erroneously formed during the second meiotic division, or post fertilisation non-disjunction of the Y chromosome (Epstein 1986, Gardner and Sutherland 1989). The incidence is not related to maternal age (Connor and Ferguson-Smith 1991). Affected individuals are tall and have a tendency toward aggressive behaviour (Hook 1973, Valentine 1986). Fertility is usually unimpaired and surprisingly the offspring have normal sex chromosomes, although there are reports of XYY males producing XYY sons (Sundequist and Hellstrom 1969). The recurrence risk in a future pregnancy is probably not increased (Gardner and Sutherland 1989).

47,XXY (Klinefelter syndrome)

This occurs in 1 in 500 males and the birth incidence is related to maternal age (Williams 1974). The extra X chromosome is of maternal origin in 60% cases and paternal origin in 40%, due to non-disjunction at meiosis (Boue and Gallano 1984). Affected individuals are tall, have hypogonadism with poor secondary sexual development and infertility (Klinefelter et al 1942). They may also suffer with scoliosis, chronic bronchitis or diabetes mellitus (Smith 1982). Affected males may be of average intelligence or have mild to moderate retardation (Modell and Modell 1992).

47,XXX (Superfemale)

This occurs in 1 in 1,000 newborn females and was first described by Jacobs et al (1959). The incidence in pregnancy increases with maternal age (Hamerton 1971). It is due to non-disjunction on either the maternal or paternal side and there are usually no clinical features, although up to 25% may have mild mental retardation (De la Chappelle 1983). Fertility is normal in the majority of cases (De la Chappelle 1983). There is no increase in recurrence risk in future pregnancies, but there are reports of other sex chromosome anomalies in offspring (Guzman-Toledano et al 1976).

45,X (Turner's syndrome)

The incidence is 1 in 5,000 female livebirths, although in pregnancy it is much higher, almost all aborting spontaneously (Lindsten 1963). It usually arises from parental non-disjunction; in 80% cases the X chromosome present is maternally derived indicating an error in the fertilising sperm or a post fertilisation error (Valentine 1986). Many cases of Turners syndrome

surviving into the postnatal period involve some form of mosaicism due to post-zygotic errors or structural abnormality of the X chromosome such as iso(X), ring(X) or deleted X. Affected individuals present at birth with nuchal oedema and peripheral lymphoedema or later in life with short stature, wide spaced nipples, streak ovaries, absent secondary sexual development and infertility (Turner 1938, Weiss 1971, Brook et al 1974). Congenital heart disease (especially coarctation of the aorta or atrial septal defects) is present in about 20% cases, intelligence is normal as is lifespan, but there is a tendency to develop systemic hypertension (Smith 1982).

Polyploidy

This is extra multiples of 23 chromosomes, instead of the normal diploid chromosome number of 46, there may be triploidy (69 chromosomes), tetraploidy (92 chromosomes) or greater. Higher orders are more likely to end in spontaneous abortion (Sheppard et al 1982). Polyploidy arises from fertilisation of a diploid ovum due to failed extrusion of the polar body at meiosis, or a diploid sperm fertilising a haploid ovum; occasionally it can be the result of polyspermic fertilisation (Jacobs et al 1978). Triploidy involves 2% of all conceptions, but survival to term is exceptional (Wertelecki et al 1976, Smith 1982). Pregnancy loss, severe asymmetrical intrauterine growth retardation and in rare survivors low birth weight are usual presentations (Wertelecki et al 1976). Multiple congenital abnormalities including syndactyly are common, and there is a 50% chance of associated hydatidiform change in the placenta (Wertelecki et al 1976, Poland and Baillie 1978). The recurrence risk is probably not increased in future pregnancies (Smith 1982, Gardner and Sutherland 1989).

1.1.4 Socioeconomic consequences

Falling perinatal mortality (Butler and Alberman 1969) and greater expectations from health care make the disappointment of having an abnormal baby worse now than ever before (Drotar et al 1975). The introduction of prenatal diagnosis offers the prospect of prevention but, it also alters the experience of pregnancy, providing a much wanted choice for some whilst for others it introduces unwanted pressure to prove their baby is normal (Rothman 1986, Lilford 1989). Consequently, the uptake of prenatal diagnosis is variable and has been shown to depend on couples perception of having an affected child (Marteau et al 1991), their attitude to termination (Marteau et al 1991) and their fear of miscarriage (Marteau et al 1989). It is also dependent on their doctors knowledge and attitude toward prenatal testing (Lipmann-Hand and Piper 1981).

The inevitable distress (Brown 1989) an abnormal karyotype result causes can be limited by support from friends and relatives, and by earlier termination of pregnancy (Iles 1989, Black 1989). Explanation that a fetal abnormality is incompatible with life can also reduce the disappointment (Iles 1989, Black 1989). A few couples choose to continue the pregnancy, even in the face of certain neonatal demise, and for them this is a preferable option (Watkins 1989) which should be supported rather than require constant justification to health professionals (Farrant 1985,Whelton 1990).

The unexpected birth of an abnormal baby may occur in unscreened younger mothers or older mothers who decline prenatal testing. The diagnosis may be obvious at birth or be delayed until confirmatory results are available. Whenever parents are informed, the shock they feel is influenced by who

explains the diagnosis and the setting in which they are told (Gath 1978). The parents may experience self blame, isolation and rejection of their abnormal child (Wolfensberger 1967, Carr 1975). The latter emotion may result in the baby's adoption, in which case the child will be fostered or remain in state care until suitable parents are found (Gath 1978).

As a direct result of the constant stress parents experience when coping with the additional demands of their handicapped child, marital relationships (Farber and Jenne 1963) and their health may suffer (Holt 1957, Schonell and Watts 1957). This effect can be significantly reduced by support from professional bodies, such as regular visits to a clinic where other mothers bring their handicapped children (Caldwell and Guze 1960). Siblings may be adversely affected in up to 15% families (Holt 1957), and this may influence decisions to limit family size (Fraser and Latour 1968) or to consider institutionalisation for the affected child (Graliker et al 1962). To minimise difficulties encountered, a sympathetic and well coordinated system of care is required from the primary health care team, hospital specialists and involved schools (Carr 1975).

The economic impact of chromosomal abnormality comprises the cost of detection (prenatal diagnosis), cost of prevention (termination of pregnancy) and the cost of care for affected individuals (Henderson 1991). The latter is accounted for by the cost of special education, health care, institutionalisation and lost income from parents and the individual (Henderson 1991). The cost therefore varies with age; for a child with Down's syndrome in 1976 this was estimated as £5,000 per year in infancy rising to over £120,000 per year in middle age (Hagard and Carter 1976). Obviously these costs would be higher now. Also the calculations vary between individual cases, for example the

loss in earning potential is related to the degree of intellectual impairment. Conley (1973) estimated that in mildly retarded individuals (IQ; 50-70) earning ability was the same as the general population until age of twenty five years, after which it fell to a low of 86%. Whilst in moderately severely retarded individuals (IQ; 40-49) earning potential was reduced to 20% of the normal population, and severe cases may not have any earning capability.

The cost of detection of chromosomal abnormality has been carefully evaluated with reference to maternal age. As the incidence of most chromosomal abnormalities increase with maternal age the cost of detecting one case (amniocentesis or CVS) will be lower in older mothers where there are more cases per 1,000 pregnancies. Conversely, the cost will be higher in younger mothers, where there are less cases per 1,000 pregnancies. Thus the cost of detection per case falls with advancing maternal age. The tangible saving of preventing birth of an affected individual can be balanced against this cost of detection. Thus an age related "cut off " can be calculated. Below this "cut off" the cost of detection of one case outweighs the saving from preventing birth of one case, this cut off has been calculated as a maternal age of 35 years (Hagard and Carter 1976, Henderson 1987), and can be used in determining the local provision of prenatal diagnostic services. The introduction of new methods of screening for chromosomal abnormality (section 1.2) will influence this cost-benefit analysis, but in which direction and by how much, remains speculative.

None of the economic calculations can take into account the intangible costs (maternal anxiety about screening, distress over miscarriage, risk of fetal damage by diagnostic procedure) and benefits (wider choices, reassurance, avoidance of distress of having an affected child) of preventing

chromosomal abnormality, so it is still not possible to determine the cut off age where prenatal diagnosis can be deemed truly efficient (Henderson 1991).

1.2 Screening for fetal chromosomal abnormalities

A screening programme must fulfil certain fundamental criteria to be effective. The disorder sought must be clearly defined, frequent enough to warrant screening for, the screening should be sensitive, highly specific and there must be an advantage in detection of the disorder (Wilson and Jungner 1968).

Fetal chromosomal abnormalities are certainly clearly defined and frequent enough to warrant screening for, but do the existing screening programmes fulfil the above requirements? The parameters suitable for a screening programme are maternal age, family history, maternal serum biochemistry and fetal morphology on ultrasound examination. After screening, the high risk mother will be offered an invasive diagnostic procedure, most commonly amniocentesis.

1.2.1 Maternal age

The risk of most chromosomal abnormalities (autosomal trisomy, 47,XXY, but not 45,X and 47,XYY) increases with advancing maternal age (Table 1.1). However, since the number of women having children decreases with advancing maternal age, only a minority are in the high risk group.

Therefore if a screening age of ≥35 years is chosen only 35% of trisomy 21 pregnancies could be detected, with a false positive rate of 7.4% (Cuckle and Wald 1987). The false positive rate will fall to only 1.1% if 40 years is chosen as the cut off, but the detection rate drops to 16% (Cuckle and Wald 1987). Furthermore, these theoretical detection rates assume 100% uptake of amniocentesis, whereas in practice only about 50% of "at risk" women choose to have a diagnostic test. In England and Wales between 1974 and 1987 the proportion of births with Down's syndrome prevented by prenatal diagnosis has remained at approximately 14% (Cuckle et al 1991). Therefore the policy of using maternal age alone as a risk factor for screening has not been successful.

1.2.2 Family history

The chance of a subsequent pregnancy being affected with a chromosomal abnormality after the birth of an affected child is only slightly increased above the age-related risk if the parents have a normal genotype (section 1.1.2). However, if the parents carry a balanced chromosomal rearrangement then the risk may be substantially increased (Table 1.3). As this situation only occurs rarely, parental karyotype is not a suitable method of screening for the general population, but does warrant an invasive procedure in affected individuals.

Parental rearrangement	Carrier	Risk of unbalanced offspring at amniocentesis (%)
Centric fusion 13;14	Father	at least 1
Centric fusion 13;14	Mother	at least 1
Centric fusion 14;21	Father	1
Centric fusion 14;21	Mother	15
Centric fusion 21;22	Father	5
Centric fusion 21;22	Mother	10
Centric fusion 21;21	Father	100
Centric fusion 21;21	Mother	100
Reciprocal (any)	Father	12
Reciprocal (any)	Mother	12
Pericentric inversion*	Father	4
Pericentric inversion*	Mother	8

*excluding pericentric inversion of chromosome 9

Table 1.3 Risks of chromosomally unbalanced offspring related to specific parental chromosomal rearrangements (Connor and Ferguson-Smith 1991).

1.2.3 Maternal serum biochemistry

Alpha-fetoprotein

The maternal serum level of alpha-fetoprotein is reduced in the second trimester of Down's syndrome pregnancies when compared to normal pregnancies (Merkantz et al 1984). Although there is considerable variation with both gestation and individual laboratory technique, this difference can be exploited as a screening tool. To overcome these variations the values are expressed as multiples of the median of an unaffected pregnancy. Despite this approach, the wide spread of values and the extensive overlap between normal and affected pregnancies, mean it is a poor screening test on its own, but it can be improved by combination with maternal age. The increased likelihood of a pregnancy being affected is calculated by the height of the

distribution curve of alpha-fetoprotein in affected pregnancies at the given value divided by the height of the distribution curve in unaffected pregnancies at the same value, and is known as the likelihood ratio (Figure 1.5). This is multiplied by the maternal age related risk to produce the new risk of the pregnancy being affected. This combination improves the detection rate over maternal age alone by about 5% without increasing the false positive rate (Cuckle and Wald 1990). The precision of this risk can be further improved by confirming gestational age with ultrasound (Cuckle and Wald 1987) and correcting for maternal weight (Cuckle,Wald and Thompson 1987).

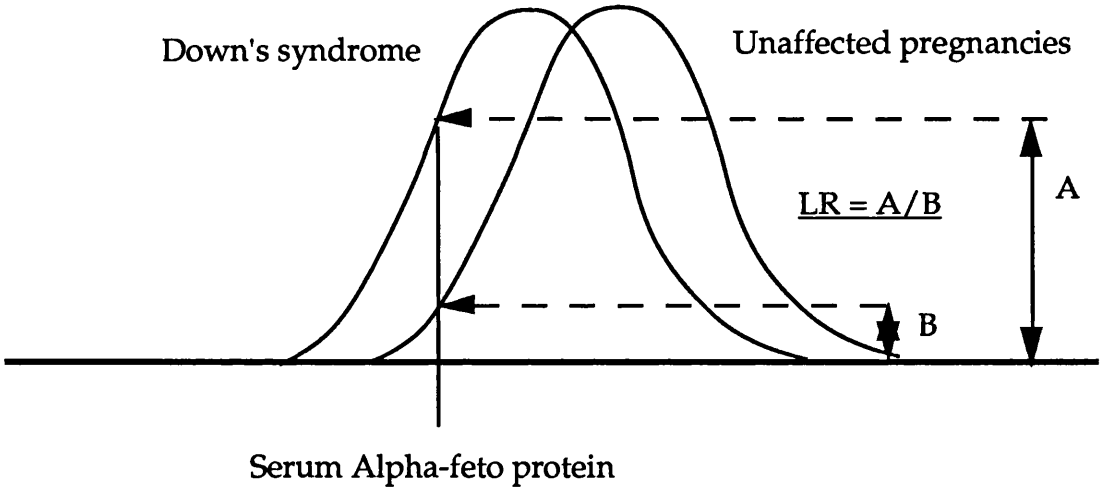

Figure 1.5 Diagrammatic representation of the distribution of maternal serum alpha-fetoprotein protein in Down's syndrome and unaffected pregnancies, illustrating the likelihood ratio (LR; A/B).

Alpha-fetoprotein levels are higher in blacks and in twin pregnancies (Wald and Cuckle 1987) and lower in insulin-dependent diabetics (Milunsky et al 1982), making risk assessment difficult. A better estimate can be made by

using race specific tables for black women, dividing the multiple of the median by 0.77 for insulin dependent diabetics and by 2 for twins (Cuckle and Wald 1990).

Unconjugated Oestriol and ß-Human Chorionic gonadotrophin

Unconjugated oestriol levels are reduced by 0.74-0.79 multiple of the median in second trimester maternal serum in Down's syndrome pregnancy when compared to controls (Canick et al 1988, Norgaard-Pedersen et al 1990). A rise of 2.08 multiples of the median has been demonstrated for ß-human chorionic gonadotrophin in the same situation (Cuckle and Wald 1990), both changes are independent of maternal age.

All three variables show little correlation, so their combination into a trivalent screening test will represent an improvement over their individual use (Wald et al 1988). The likelihood ratio is calculated for each of the three factors (as above) and multiplied by the maternal age related risk to provide the new risk estimate. The detection rate can be selected dependent upon what false positive level is acceptable (Table 1.4) and what resources are available for invasive testing to confirm or refute high risk cases. Accepting a 5% false positive rate, 61% of Down's syndrome cases would be detected, making this the most efficient (highest sensitivity and specificity) screening test available.

False positive rate (%)	Detection rate (%) using age with:			
	AFP	uE3	hCG	AFP,uE3 and hCG
1	18	21	28	38
2	24	27	36	47
3	28	32	41	53
4	32	36	45	57
5	34	40	49	61
6	37	43	52	64
7	39	46	55	66
8	42	48	57	69
9	44	50	59	71
10	46	52	61	72

Table 1.4 Detection rate of Down's syndrome in relation to false positive rate using maternal age with alpha-fetoprotein (AFP), unconjugated oestriol (uE3) and beta human chorionic gonadotrophin (ß-hCG) individually, or in combination (Cuckle and Wald 1990).

One disadvantage of this triple test is the late stage at which it is performed (16 week's gestation) which means that results from a subsequent amniocentesis will not be available until about 20 week's gestation. A termination of pregnancy at this stage is not only extremely distressing to all concerned but also technically more difficult and carries a higher morbidity than in the first trimester. However, there is some evidence to suggest that similar changes in alpha-fetoprotein and unconjugated oestriol are present in the first trimester (Mantingh et al 1989, Cuckle et al 1988) and may be suitable for an earlier screening test; this does not appear to be true for beta human chorionic gonadotrophin (Bogart et al 1989).

Another disadvantage is that the test is based on detection of trisomy 21 pregnancies only and does not directly screen for other chromosomal abnormalities, although there is some evidence that trisomy 18 pregnancies

may be detected (Palomaki 1986).

Improvement of this screening test would be its application in the first trimester and evidence of its ability to detect other chromosomal aneuploidy as well as Down's syndrome. The success of the test depends on the way it is introduced and the way the health professionals and parents interpret the results. This latter point will also influence the number of amniocenteses which will be performed as a result of triple screening.

1.2.4 Ultrasonography

The characteristic clinical features of Down's syndrome enable the diagnosis to be made in the newborn (always subject to chromosomal confirmation). As these features are also present in a proportion of the affected fetuses, ultrasound examination introduces the possibility of making the diagnosis prenatally. Brachycephaly, nuchal thickness, cardiac anomalies, relative shortening of the femur, hydronephrosis, camptodactyly and sandal gap can all be diagnosed by ultrasound examination at about 20 weeks gestation (Nicolaides et al 1992a). Visualisation of these abnormalities increases the operators suspicion of chromosomal abnormality and raises the question of an invasive test (amniocentesis, cordocentesis or placental biopsy) to confirm the diagnosis. However the sensitivity and specificity of this as a screening test is not known and will be subject to inter-operator variation. Some authors have tried to use fetal measurements to display the same information and add a degree of objectivity. The cephalic index (ratio of the fetal biparietal diameter to occipito-frontal diameter) has been used to establish if brachycephaly is present, but five studies involving 78 affected

pregnancies have failed to demonstrate a useful association (Perry et al 1984, Lockwood et al 1987 and Brumfield et al 1989).

The measurement of nuchal thickness in the second trimester initially offered encouraging detection rates (43% of Down's syndrome) with a low false positive rate (0.1%; Benacerraf, Frigoletto and Cramer 1987). However, the heavily subjective assessment has not resulted in reproducible results, as inadvertent angulation of the transducer or extension of the fetal neck can both produce false positive measurements.

Short stature is a feature of postnatal Downs syndrome, so could prenatal femur length measurement be used as a screening test? Initial reports suggested it could, demonstrating significant shortening in affected pregnancies (Benacerraf, Gelman and Frigoletto 1987, Lockwood et al 1987), but later reports found either no effect (Platt et al 1988, Lynch et al 1989) or minimal difference (Peters, Lockwood and Miller 1989, Cuckle 1989). Evidence from the largest study suggests that femur length measurement would result in a 34% detection rate at a 5% false positive rate; similar to maternal age alone (Cuckle 1989).

The disadvantage with ultrasonography, as with biochemical testing, is the late gestation at which the test can be performed. However, recent experience of anomaly scanning in earlier pregnancy has demonstrated a valuable finding; nuchal translucency. This is a fluid filled membrane seen extending around the fetal occiput and neck in 6% of pregnancies in the first trimester (Nicolaides et al 1992b). If the thickness of this membrane is $\geq$3 mm or greater the risk of chromosomal abnormality is ten times the maternal age related risk. If there is no membrane or it is less than 3 mm the risk is

reduced to one third of the age-related risk (Nicolaides et al 1992b).

Thus, with the exception of this last finding it appears that ultrasound is not an improvement over biochemical screening, for the detection of chromosomal abnormality. The precise risk of chromosomal abnormality in the presence of ultrasound detected abnormalities is still to be established.

1.3 Diagnostic techniques in the second and third trimester

These are essentially amniocentesis at 16 weeks, late placental biopsy and cordocentesis. As the subject of this thesis is amniocentesis, this section summarises the development and historical background of the technique.

1.3.1 Amniocentesis

In 1882 Friedrich Schatz published a report on twin pregnancies in which he described the experience of one of his colleagues, who aspirated what he believed to be an ovarian cyst complicating pregnancy. It was, in fact a twin pregnancy with polyhydramnios and the procedure he inadvertently described was the first record of amniocentesis.

The first author to explain his technique for amniocentesis was Henkel in 1919, who performed the procedure in a case of polyhydramnios. In subsequent years it was used with success as a method of treatment for both acute and chronic polyhydramnios (Rivett 1933). This was the only

indication for amniocentesis until 1930, when Thomas Menees et al described the technique of amniography (Menees et al 1930). The procedure involved passing a flexible lumbar puncture needle through the abdominal and uterine walls, in the midline. Amniotic fluid was aspirated to confirm position and USP Strontium Iodide was injected; thirty minutes later the abdomen was X-rayed and the fetus visualised, contrasted against the radio-opaque amniotic fluid. This enabled visualisation of the placenta, fetal parts, fetal position, and occasionally fetal sex. The authors suggested it was useful in diagnosing placenta praevia and described 21 uncomplicated procedures. Unfortunately, the only case of placenta praevia in their amniography series ended in perinatal death after a placental abruption. Despite such setbacks the procedure was adopted into clinical use and the technique modified by other authors (Cornell et al 1933, Aburel 1937).

A new indication for amniocentesis was introduced in 1952 when Bevis discovered that biochemical changes in amniotic fluid were related to the severity of rhesus isoimmunisation in affected pregnancies. The initial investigations were performed on liquor obtained after rupture of membranes at term, but subsequent samples were collected by "abdominal paracentesis" from 28 weeks gestation. A 20 gauge spinal needle was inserted sub-umbilically in the midline and 3 ml of amniotic fluid aspirated. Bevis described the prognostic value of measuring amniotic fluid levels of non-haematin iron and urobilinogen in the management of rhesus immunised pregnancies. In 1953, Bevis described the association between the severity of fetal haemolytic disease and blood pigment levels in the amniotic fluid. This work involved the collection of amniotic fluid from 22 weeks gestation, earlier than previously reported. Of the 150 patients who underwent amniocentesis he reported only one spontaneous abortion, at 24

weeks, of an "abnormal" fetus. In his paper the author stated that amniocentesis before 22 weeks is usually impossible (Bevis 1953), thus defining the practical lower limit of the procedure, at the time.

Although the number of amniocenteses performed increased as a result of its use in the management of rhesus disease, it was still only performed in a minority of pregnancies. No increase could be expected unless a wider indication for the technique were unearthed.

The ability to determine sex by staining sex chromatin in desquamated cells was described in 1955 (Moore and Barr 1955). This paved the way for application of the technique to amniotic fluid cells, to determine fetal sex antenatally. By the end of 1955 and within five weeks of each other, four papers were submitted describing chromatin staining in amniotic fluid cells (Fuchs and Riis 1955, Shettles 1955, Serr et al 1955, Makowski et al 1955). Although initially some authors were dubious about the clinical role of the test (Fuchs and Riis 1955), it represented a major step toward current prenatal diagnosis. Fuchs and Riis collected amniotic fluid from term pregnancies at induction of labour, when the membranes were ruptured, and in mid pregnancy from surgical terminations. The fetal sex was determined by staining for sex chromatin (Moore and Barr 1955). They successfully diagnosed the fetal sex in 20 out of 21 cases, the only failure was attributed to inexperience. They demonstrated that there were sufficient numbers of cells to allow successful testing, even though examination of a high proportion of cells was impossible because they were dead, damaged or pyknotic. Makowski et al (1956) stained cells from 30 pregnancies and showed that there was no overlap between the mean number of chromatin positive cells in females and males, indicating the high specificity of the test (Table 1.5).

The only incorrect diagnosis was made in a case where the amniotic fluid was contaminated with maternal cells and faeces, due to a recto-vaginal fistula.

	Observer 1			Observer 2		
		Chromatin positive cells			Chromatin positive cells	
sex	Number	Range (%)	Mean (%)	Number	Range (%)	Mean (%)
Male	14	6 - 17	10	6	6 - 22	13
Female	16	49 -71	59	6	42 - 70	54

Table 1.5 Percentage of sex chromatin positive cells in amniotic fluid from male and female fetuses, as examined by two different observers (Makowski et al 1956).

These early reports were unanimous about the reliability of the technique for prenatal sex determination, but were less clear about the indication for the test. In 1960, Riis and Fuchs published their experience with the technique in two haemophilia carriers. They performed transvaginal amniocentesis in the second trimester, in one case they successfully obtained a sample and diagnosed a female fetus, in the other they were unsuccessful, so repeated the procedure transabdominally. They obtained a sample and diagnosed a girl also. The first pregnancy ended in premature labour at 28 weeks gestation, but after neonatal intensive care the baby survived without morbidity. Unfortunately, the second pregnancy ended in an intrauterine death at 29 weeks, of unknown cause (Fuchs and Riis 1960).

Apart from the above attempts at amniocentesis in earlier pregnancy, the

majority of procedures were performed close to term, as sampling was more successful in later pregnancy. However, in 1958 Parrish et al published their experience of 50 amniocenteses from 20 weeks gestation. They suggested that the term "transabdominal amniocentesis" be universally adopted to describe the technique, and phrases like abdominal paracentesis and paracentesis uteri be abandoned. They reported that the procedure was not performed frequently because most clinicians did not appreciate how simple it was. They detailed their technique which involved passing an 18 gauge needle transabdominally through sterile abdominal skin at a point midway between the umbilicus and symphysis pubis. They catheterised the patient first, and with the first six cases gave the mother premedication. After these cases they discontinued premedication using only local anaesthetic, injected down to the level of the peritoneum. Their success rate by gestation is shown in Table 1.6. There were no fetal or maternal complications, and although the authors considered the technique safe they recommended further studies.

The risks associated with amniocentesis were hotly debated, with views ranging from total confidence in its safety, to extreme concern about its dangers and limited indication (Liley 1960). Both were extreme views, but there was no clear evidence to indicate what the procedure-related complications and their incidence were. As, at the time, it was performed in pregnancies at high risk of fetal loss (rhesus immunised fetuses) an element of procedure related risk was considered justified. However, this view would have to change if amniocentesis were to be used for lower risk indications.

Gestation (weeks)	Number of cases	Number of successful taps (%)
20 - 23	5	5 (100)
24 - 27	8	8 (100)
28 - 31	13	12 (92)
32 - 35	13	11 (85)
36 - 40	11	10 (91)
Total	50	46 (92)

Table 1.6 Gestation and number of successful amniocenteses reported (Parrish et al 1958).

In 1966, when Steele and Bregg described their technique for culturing fetal cells from amniotic fluid in sufficient quantities to allow karyotyping, the doors for widespread prenatal diagnosis were opened. The diagnostic possibilities of the procedure were confirmed when Nadler (1968) diagnosed Down's syndrome on amniotic fluid obtained at termination of pregnancy at 10 weeks' gestation. The following year Nadler (1969) reported diagnosing translocation trisomy 21 by amniocentesis at 16 weeks, where the mother was a balanced translocation carrier. The pregnancy was terminated and analysis of the abortus confirmed Down's syndrome. As a result of these discoveries the indications for amniocentesis broadened greatly, to include those at risk of chromosomally abnormal pregnancies. Initially, only those at high risk of chromosomal abnormality, such as parental balanced translocation, were tested but as experience with the technique grew, it was offered to mothers with relatively low risk indications, such as advanced maternal age. As a consequence of this diversification, determining the procedure related risk

became more important than ever, as a small procedure related risk might be greater than the risk of having an affected pregnancy. Genetic counselling of parents seeking amniocentesis depends on the balance of these risks and their interpretation of this balance.

Initially, the risk was investigated by retrospective review of reported series such as by Burnett and Anderson in 1968 who reviewed 8,300 procedures reported between 1933 and 1966. The procedures were performed for amniography, rhesus disease or to relieve polyhydramnios, and resulted in 11 fetal losses, two cases of chorioamniitis and one placental abruption. Surprisingly low morbidity for an invasive procedure performed blindly; the authors concluded that complications from amniocentesis were rare but could be reduced still further if certain guidelines were adhered to. They suggested that the needle should be removed if blood were aspirated and that drainage of polyhydramnios should be gradual to prevent placental abruption. It is impossible to establish the true risk of the procedure from these reports, as they were performed using different methods, many patients were not followed up and not all procedures were included.

Despite the lack of data on safety, the widening indications for amniocentesis (Table 1.7) resulted in an exponential increase in the number of procedures performed. By the mid 1970's, the majority of practising Obstetricians were familiar with the technique (Schwarz 1975). The introduction of B mode ultrasound scanning into Obstetrics provided the opportunity to localise the placenta prior to needle insertion in an effort to reduce the fetal risks. Although, initially this was considered to be of little benefit as complications were so infrequently reported without the use of ultrasound (Schwarz 1975), it was adopted by many. As complications were rare a large study would be

required to establish any true benefit of ultrasound, and no such study had been performed.

Indication for amniocentesis	Author	Year of first report
Amniography	Menees et al	1930
Drainage of polyhydramnios	Rivett	1933
Abortion by intramniotic injection of hypertonic solution	Aburel	1937
Assessment of Rhesus isoimmunisation	Bevis	1950
Fetal sex determination	Riis and Fuchs	1956
Measurent of amniotic fluid gas tensions	Sjostedt et al	1958
Assessment of Rhesus isoimmunisation	Liley	1963
Fetal kayrotyping	Steele and Bregg	1966
Amniotic fluid Oestriol	Berman et al	1968
High maternal serum AFP for fetal neural tube defect	Brock and Sutcliffe	1972
Amniotic fluid enzyme levels	Kaback et al	1972
Low maternal serum AFP for chromosomal abnormality	Merkatz et al	1984

Table 1.7 Indications for amniocentesis.

In 1976 the first large prospective controlled trial of amniocentesis was published (NICHD National Registry for Amniocentesis Study Group 1976) in which 1,040 women undergoing amniocentesis were compared to 992 controls. A control patient was sought at the time the subject had her amniocentesis and was matched for age (+/- 2 years), race, income, gestation and parity. Unfortunately this was not always possible and nearly a quarter (24%) of subjects remained unmatched. As a result the subject group were older and differed in gestation at trial entry. The majority (79.6%) of amniocenteses were performed for a low risk indication (maternal age, previous affected child or family history, in the absence of parental chromosomal rearrangement). A result was not always obtained at the first attempt, repeat taps were needed in 136 cases (13%), due mainly to failed sampling (45%) or failed culture (43%). In three cases of failure to obtain a

result the mother declined a repeat test, one had a normal outcome, one resulted in an affected haemophiliac male and the third in a child with trisomy 21.

Abnormal karyotype was present in 1.8% of cultures, 1.4% revealed a metabolic abnormality and 1% revealed a male fetus in mothers carrying X-linked disorders, making a total of 4.2% positive diagnoses. There were six erroneous diagnoses (0.6%), four falsely positive and two falsely negative. The latter two indicated normal karyotypes, but a baby with Trisomy 21 was delivered in both cases.

The overall fetal loss rate was 3.5% (spontaneous abortions, intrauterine death and stillbirths) in the amniocentesis group and 3.2% in the controls. When the differing maternal age in the two groups was controlled for, the fetal loss rates changed to 3.3% and 3.4% for the control and amniocentesis groups respectively. Neonatal outcome was obtained for 93% of the amniocentesis group and 96% of the controls. There was no significant difference in; preterm delivery rate, pattern of congenital abnormalities, infant deaths in the first year or infant growth, between the two groups. The authors concluded that amniocentesis was a safe procedure and that the generally accepted 1% fetal loss rate was exaggerated. They also concluded that their reassuring results would allow clinicians to offer amniocentesis to all mothers over 35 years of age. They expressed their concern about the false diagnoses, which they were unable to explain but thought the most likely explanation was maternal cell contamination.

It is unfortunate that such a large proportion of cases were unmatched as it introduced bias into the sample groups. There is no mention of the length of

delay between enrolling the subject and the control. Ideally there should be no delay, controls being matched either before or at the time of the amniocentesis. Any delay in finding a control introduces bias, as events may occur in this time which will not be recorded. Consequently the findings may not be representative. This is the first report to quantify errors in diagnoses and illustrate their potential disastrous effect. Two out of three of the failed sample who declined further investigation had an affected pregnancy, underlining the need to repeat tests in such cases.

In 1978 the working party on the hazards of amniocentesis reported their findings to The Medical Research Council (MRC). The report (MRC 1978) was the result of a prospective non randomised case controlled study and a supplementary study. Nine centres collaborated and all but two centres entered all their amniocenteses performed during the 44 months of the study. After an amniocentesis was performed a search was made for a control, matched for age (within 3 years), gestation, obstetric history and rhesus status. Unfortunately review of the data after 30 months demonstrated that the long delays in finding a control had introduced bias between the groups (the criticism of the NICHD study). After interim data review the remainder of the study was performed with the control being identified before the subject underwent amniocentesis.

197 women were excluded from the subject group, leaving 2,934, of whom 17% had unmatched controls. The indication for the procedure was for fetal karyotyping in 52% (of which 73% were for maternal age) diagnosis of neural tube defect in 41% and other reasons in 7%.

The fetal loss rate in the main study (in matched subjects) was 1.3% greater

than controls, in the supplementary study it was 1.5% greater. The perinatal mortality was 1.1% greater in subjects than matched controls. There was a significant increase in stillbirths and neonatal deaths in the amniocentesis group and the incidence of unexplained respiratory difficulties and postural limb deformities was significantly higher in the amniocentesis group (Table 1.8).

	Amniocentesis	Matched controls
Unexplained respiratory difficulties	1.2%	0.4%
Postural deformities	2.4%	1.1%
Stillbirths	0.8%	0.4%
Neonatal death	1.0%	0.4%

Table 1.8 Incidence of selected complications after amniocentesis (MRC 1978).

The conclusions of the study were that amniocentesis from 16-20 weeks gestation may be associated with increased risk to the outcome of the pregnancy. The hazards are miscarriage, premature rupture of membranes, antepartum haemorrhage and rhesus isoimmunisation. The neonate is at increased risk of unexplained respiratory difficulty and postural limb deformities. Neonatal postural limb deformity was not increased in the supplementary study where cases were matched before amniocentesis, indicating that this may have been a chance finding. The increased fetal loss rate of 1.3-1.5% with amniocentesis is in conflict with findings of the NICHD study.

The major criticisms of the main study is that not all the controls were matched at the same time as the subject entered the study. Unless the groups are matched at the time of the procedure, potential controls who miscarried soon after would not be included, and only women with ongoing pregnancies selected, thus falsely increasing the miscarriage rate in the amniocentesis group over the controls. The working party recognised this ascertainment bias and initiated the supplementary study to correct the situation. The methodologically correct supplementary study also demonstrated increased fetal loss after amniocentesis, indicating that it probably is a true finding. Another criticism of the study is that all indications for amniocentesis were included. Thus the group constituted high (carriers of genetic abnormalities) and low risk cases (advanced maternal age). Background miscarriage rates in these groups may differ significantly, and not be similar to the control group.

Ultrasound was used in a minority of cases to localise the placenta, and by one clinician to guide the needle into the amniotic sac. The numbers were too small to demonstrate any correlation with pregnancy loss, but ultrasound did reduce the number of needle insertions required. Without prior ultrasound more than one needle insertion was required in 18.7% cases, with ultrasound this fell to 16.4% and with synchronous ultrasound guidance it was only 5.6%. As ultrasound was not routinely performed in the control group or in the majority of subjects it is possible that non viable pregnancies were included in the study. The biased use of ultrasound toward the amniocentesis group would have eliminated some non viable pregnancies prior to the procedure, reducing the proportion of fetal loss in this group.
Although this study addressed the safety of amniocentesis in great detail it was not designed in the best way to answer the question of safety and the bias

discussed above diminishes the significance of the results. The best study design is a prospective randomised controlled study in a low risk population, with randomisation taking place either before or at the time of the amniocentesis.

Subsequent to the MRC study a series of trials were published (Table 1.9), of which the best designed came from Denmark (Tabor et al 1986), a prospective randomised controlled trial in a low risk population. 4,606 low risk women (25-34 years old) were randomised at the same gestation to receive amniocentesis or not. All patients (subjects and controls) received an ultrasound scan and the amniocentesis was performed with continuous ultrasound guidance in all cases. The randomisation resulted in two directly comparable groups, compliance with allocated grouping was 99.1% for the amniocentesis group and 90% for controls. All cases were followed up to the neonatal period. The results demonstrated a 1% increase in spontaneous abortion after amniocentesis (1.7%) over controls (0.7%) and a 1% increase in neonatal respiratory complications (1.8% versus. 0.8%). The study demonstrated an increased risk of spontaneous abortion if the placenta was punctured during amniocentesis. Also the risk of abortion was increased if serum alpha-fetoprotein was raised, the amniotic fluid was discoloured or there was post-procedure leakage of liquor. In contrast to the MRC study, Tabor et al found no difference in rates of antepartum haemorrhage or neonatal postural deformities between the groups. The findings of this study are most likely to represent the true risk of amniocentesis, as the groups were comparable, randomisation was correctly applied and follow up was complete.

Thus, in experienced hands the procedure carries a 1% risk (additional to

background risk) of causing spontaneous abortion, and indications for the procedure should be weighed against this statistic.

Although the precise mechanism of pregnancy loss after amniocentesis is unclear, it is unlikely to be due to one specific cause, more probably it occurs for a variety of different reasons. Pregnancy loss is highest within three weeks of the procedure (Tabor et al 1986), suggesting a direct effect from the amniocentesis, such as trauma from the needle, uterine contraction or infection. However, the increased loss is detectable for up to 64 days after sampling (Tabor et al 1986), which is more difficult to explain. Needle trauma to the fetus occurs in a minority of cases, but even in cases of severe damage, the pregnancy usually continues (Broome et al 1976, Merin and Beyth 1980). Needle trauma to the placenta and umbilical cord, can result in fetal demise, and is probably a greater cause of pregnancy loss. If fetal or placental haemorrhage occurs during amniocentesis and ultrasound has not been used, not only will the operator be unaware of the complication but also the cause of subsequent pregnancy loss will be unknown.

Study	Year	Design	Number of patients		Amniocentesis complications					Cytological details			
			Subjects	Controls	Low risk cases	>1 needle insertion	Amniotic fluid leakage	Vaginal bleeding	Fetal loss rate	Abnormal karyotype	Culture failure	False positive	False negative
NICHD	1976	Non random cohort	1,040	922	80%	13%	1.1%	1%	+0.3% (3.5 vs 3.2)	1.8%	4.5%	0.4%	0.2%
Simpson et al	1976	Series	1,020	—	50%	24%	—	—	3.3%**	2.9%	3.7%	0.25%	0.0%
MRC	1978	Non random cohort	3,131	2,428*	39%	7%	—	+0.9%	+1.5%*** (2.6 vs 1.1)	1.5%	4.0%	—	—
Crandall et al	1980	Retrospective non random	2,000	2,000	85%	3%	0.25%	—	+0.2% (2.7 vs 2.2)	—	2.2%	—	—
Bartsch et al	1980	Series	1,000	—	92%	9%	—	—	1.8%	2.2%	—	—	0.6%
Verjaal et al	1981	Series	1,500	—	44%	2%	—	0.2%	1.5%	1.3%	4.3%	0.06%	0.06%
Dacus et al	1985	Series	2,000	—	82%	10%	0.2%	—	1.7%	1.0%	2.3%	0.10%	—
Terzian et al	1985	Series	4,357	—	52%	3%	—	—	3.1%	1.2%	—	0.18%	—
Leschot et al	1986	Series	3,000	—	51%	—	—	—	2.3%	—	—	—	—
Tabor et al	1986	Prospective randomised	2,286	2,291	100%	2%	+1.3%	- 0.2%	+1% (1.7 vs 0.7)	1.0%	0.5%	—	—

+ or - sign indicates difference between amniocentesis group and control
* 17% cases were unmatched
*** Includes spontaneous abortions and stillbirths
*** Figure from supplementary study. Main study fetal loss rate was +1.3% (2.7 vs 1.4)

Table 1.9 Selected studies of amniocentesis.

Transplacental amniocentesis can be associated with severe feto-maternal haemorrhage (Zipursky 1963), indicating good reason to avoid the placenta. Placental localisation prior to amniocentesis has been shown to reduce the number of needle insertions (MRC 1978) and bloody aspirates (Harrison et al 1975). As both of these latter events are associated with an increased risk of spontaneous abortion, avoiding the placenta is the best technique, as it is one of the few methods of directly reducing procedure related complication. The size of needle has been implicated as a relevant factor, with one study recommending nothing larger than an 18 gauge (NICHD 1976) and another, nothing larger than 20 gauge (Simpson et al 1976) needle. The best advice is to use the smallest needle possible, to reliably obtain a sample. Experience of the operator may be relevant, as less experienced operators have been associated with increased risk of pregnancy loss (MRC 1978) and feto-maternal haemorrhage (Leschot 1985).

Appreciation of these factors has influenced the method of amniocentesis. It was introduced prior to ultrasound and was, by necessity, a blind procedure. The introduction and rapid uptake of ultrasound into Obstetrics has resulted in the majority of operators using it during amniocentesis. There are two approaches to ultrasound use. The first is to use it to demonstrate fetal viability, placental site and an adequate "pool" of liquor. The chosen site is marked, the transducer removed and the needle inserted. The second approach is to establish the same findings, then to guide the needle into the liquor under direct ultrasound vision. The second approach is an improvement, as the fetus and needle position are constantly seen, so the latter can be adjusted if the former changes. The first method does not use the full potential of ultrasound and should not be called ultrasound guided amniocentesis.

Amniocentesis: summary

1. Amniocentesis was first described in 1882 and has subsequently been used for the management of polyhydramnios, Rhesus disease, detection of neural tube defects and diagnosis of chromosomal abnormality.

2. At present the main indication for the procedure is fetal karyotyping in pregnancies at low risk of chromosomal abnormality.

3. Amniocentesis, when performed at 16 - 20 weeks gestation produces successful cell cultures in 96 - 99.5% cases.

4. Both false positive (0.06%-0.4% cases) and false negative (0.06%-0.6% cases) results occur with amniocentesis and may necessitate repeat testing.

5. The best evidence indicates a 1% increase in the spontaneous abortion rate after amniocentesis and a 1% increase in unexplained respiratory difficulties in the neonate.

6. Ultrasound guided amniocentesis reduces the number of needle insertions and bloody aspirates, both of which are associated with increased pregnancy loss.

7. Increased operator experience results in a decrease in post procedure complications.

1.3.2 Cordocentesis and placental biopsy

Ultrasound guided fetal blood sampling from an umbilical cord vessel can be successfully carried out from 18 weeks gestation onwards. Cytogenetic analysis from cultured lymphocytes is possible within 2-3 days of sampling. The technique is currently available in highly specialised prenatal diagnostic centres and the procedure-related fetal loss rate is reported at 1-2% (Nicolaides and Snijders 1992). The main value of cordocentesis in fetal karyotyping is (i) in the investigation of ultrasonographically detected fetal malformations, (ii) in providing a diagnosis when mosaicism is detected at amniocentesis or chorion villus sampling and (iii) rapid karyotyping for older women who book late in pregnancy.

Placental biopsy can be performed at any stage of pregnancy from six weeks gestation (Brambati et al 1991, Nicolaides et al 1986a). This technique can be performed as an alternative to amniocentesis at 16 weeks or as an alternative to cordocentesis at later gestations. The main advantage is that when a fetal abnormality is diagnosed by ultrasound, cytogenetic analysis by direct preparations can provide the result within a few hours. The disadvantage is the possibility of false positive and to a lesser extent, false negative results. The commonest chromosomal abnormality found in association with ultrasonographically detectable abnormalities is trisomy 18 (Nicolaides et al 1986b), which is the chromosomal abnormality with the highest incidence of false positive and negative results with chorion villus sampling (Gosden 1990).

Additionally and, unlike fetal blood sampling, placental biopsy will only provide information on fetal karyotype, not on any of the other causes of a

fetal abnormality, as exemplified by hydrops fetalis, which may be a consequence of chromosomal abnormality, various metabolic disorders, congenital infection or anaemia.

1.4 Diagnostic techniques in the first trimester

Chorion villus sampling was, until relatively recently the only diagnostic technique available for prenatal diagnosis of fetal karyotype in the first trimester of pregnancy. However, there are now several reports of attempts at first trimester amniocentesis, with encouraging preliminary results. This section will review both techniques.

1.4.1 Chorion villus sampling (CVS)

Development

The first description of prenatal diagnosis from trophoblast tissue was by Hahnemann and Mohr in 1968, when they reported blind biopsy of the chorionic membrane. Subsequently, Hahnemann developed a sampling method under direct hysteroscopic vision, but the technical difficulties involved hindered the procedure's acceptance into clinical practice (Hahnemann 1974).

Aspiration of chorionic villi trancervically was achieved in China (Tietung Hospital 1975), where it was successfully used for prenatal diagnosis of fetal

sex in 94% cases, by examining the cells obtained for sex chromatin. The quoted pregnancy loss rate was only 4%, despite the procedure being performed blind. Subsequently a simpler biopsy method was proposed using a throat swab to gather exfoliated cells from within the cervix (Rhine et al 1977), but the high degree of maternal cell contamination precluded its practical use.

In England transcervical CVS was successfully performed prior to termination of pregnancy, both blind and with ultrasound guidance (Horwell et al 1983). As a result of this success it was introduced into clinical practice, although there was still little data on the safety of the procedure.

Transabdominal CVS was introduced in 1984 (Smidt-Jensen and Hahnemann 1984), as an alternative to the transvaginal route. The perceived advantages were; reduced risk of fetal and maternal infection, wider gestational range for the test, greater control over size of the sample and a less difficult procedure (Lilford et al 1987). The advantages of transabdominal over transcervical sampling have since been confirmed by the significantly lower fetal loss rate in the former when both were compared to amniocentesis in a randomised study (Smidt-Jensen et al 1991).

Thus CVS provides an alternative to amniocentesis for prenatal diagnosis of fetal karyotype and offers the advantage of diagnosis in the first trimester. Using direct preparations the karyotype result can be available within 6 hours of the procedure, with 2-3 weeks for the confirmatory cultures.

Technique

Ultrasound guided CVS can be performed by transabdominal or transvaginal routes. For transvaginal CVS, the vagina is cleaned with antiseptic, and a plastic or metal cannula introduced through the cervix. The cannula is advanced into the placenta and a sample is collected either by aspiration, with a syringe or mechanical sucker and trap, or with biopsy forceps. The sample is examined and if sufficient for diagnosis (>5 grams wet weight) the cannula is withdrawn. If the sample is insufficient a further aspiration or biopsy can be collected through the same cannula.

Transabdominal CVS is performed after a careful ultrasound examination has defined the placenta and clearly demonstrated the best path for the needle. The maternal abdominal wall is cleaned with antiseptic and the needle introduced into the substance of the placenta, with or without a needle guide. The amniotic sac is always avoided. Correct needle placement can be confirmed by ultrasound, and also by the sensation where the needle can be advanced effortlessly within the placenta, in direct contrast to the resistance of the myometrium and decidua. Once the needle (18 gauge) is in place different sampling techniques can be used, either a finer needle (20 gauge) can be passed down it for aspiration of the sample, or a fine pair of forceps can be introduced and a biopsy taken. Successful sampling can been achieved with a single small needle (20 or 22 gauge) without an outer needle (Byrne et al 1991). The perceived advantage is that the smaller needle may prove to be less traumatic, whilst the disadvantage is that if the sample is inadequate the needle must be re-introduced as there is no outer needle in place. In the double needle system only the inner needle is re-introduced if the sample is inadequate. However, in experienced hands the need for repeat

needle insertion is rare with the smaller needle (Byrne et al 1991).

The sample is aspirated into culture medium containing heparin, cleaned of any blood or decidual contamination and transported to the laboratory in transport medium.

Safety and diagnostic accuracy

The procedure related pregnancy loss rate has been examined in two large multicentre randomised trials comparing the procedure to second trimester amniocentesis. The first, from Canada (Canadian Collaborative CVS - Amniocentesis Clinical trial group 1989) demonstrated a pregnancy loss (spontaneous and induced abortions plus late losses) of 7.0% (95% CI; 5.6-8.6%) in the amniocentesis group, whereas in the CVS group this was 7.6% (CI; 6.2-9.3%); a difference of 0.6%. This increase was later confirmed by the Medical Research Council European trial (Medical Research Council 1991) which showed significantly fewer surviving children in the CVS group when compared to the amniocentesis group (86% versus 91%). The difference was mainly due to the significantly higher fetal loss before 28 weeks and the larger number of terminations for chromosomal abnormality in the CVS group. The majority of the fetal losses occurred within 4 weeks of the CVS and there were more premature deliveries in the CVS group, which partly explains the increased neonatal deaths.

One of the most recent studies on the safety of CVS involved a randomised comparison between transcervical CVS, transabdominal CVS and second trimester amniocentesis (Smidt-Jensen et al 1991). The authors found no significant difference in the total fetal loss rate between the transabdominal

CVS group and the amniocentesis group (6.2% and 6.3% respectively; p=0.01), whilst there was a significantly higher fetal loss rate following transcervical CVS (10.1%).

In the Canadian study (Canadian Collaborative CVS-Amniocentesis Clinical trial group 1989) there were significantly more laboratory failures in the CVS group (1.5% versus 0.1%; p<0.001) as well as a significantly higher number of chromosomal abnormalities (4.6% versus 2.4%; p<0.01). The latter difference was explained for the most part by the significantly higher number of false positive results in the CVS group (2.0% versus 0.3%; p<0.001) due to confined placental abnormalities. In the MRC trial there were also more abnormal results in the CVS group (MRC 1991) which were also largely due to placental confined abnormality. The concern of placental confined abnormalities is that if not recognised a normal fetus may be aborted, or even an abnormal fetus be assigned a normal result. The laboratories are familiar with a proportion of these abnormalities, which decreases the number that cause a clinical problem.

Of greater concern is the possible link between fetal limb and facial abnormalities and early CVS. In a study from Oxford of 289 pregnancies where CVS was performed at 55-66 days' gestation 4 babies were born with oromandibular-limb hypogenesis syndromes (Firth et al 1991). Considering the estimated birth prevalence of this is 1 per 175,000 livebirths (Froster and Baird 1989), the high incidence raises the possibility that CVS was causative. Although it is unlikely that there will ever be sufficient data to prove this association case reports from other centres (Mastroiacovo and Cavalcanti 1991, Hsieh et al 1991, Miny et al 1991) add further credence to this possibility, indicating that it may be a related phenomenon rather than random

clustering. It is therefore, a potential risk which should be discussed with any mother requesting CVS in very early pregnancy.

In conclusion, the best available evidence suggests that CVS when compared to second trimester amniocentesis carries a greater risk of; (i) pregnancy loss, (ii) a misleading result, (iii) need for a repeat procedure and (iv) possible fetal abnormality. To these risks should be added the fact that it is a more expensive procedure both to perform and cytogenetically analyse.

In a historical context amniocentesis also suffered from serious disadvantages initially, but these were overcome with time and experience. It is impossible to know to what extent the same will be true of CVS; it is a technique which must at present remain for new methods to be compared against. The potential disadvantages of CVS must be weighed against the tremendous advantage to parents of first trimester diagnosis with a rapid result. Currently there is a demand for the procedure, but the impact of possible associated fetal limb abnormalities has not been carefully audited yet. A technique which offers the advantage of early diagnosis without the associated problems seen with CVS would represent a major step forward.

1.4.2 Early amniocentesis

Early amniocentesis differs from conventional amniocentesis in gestation only. The procedure is the same, aspiration of fluid from the amniotic sac. However the anatomical differences of the developing pregnancy in the first trimester make amniotic fluid sampling at this stage technically more demanding, primarily due to the separated amniotic and chorionic membranes, which form a double barrier to the sampling needle. The

relationship of the fetal membranes change with advancing gestation, so it is worth considering the embryology in more detail before discussing the invasive procedure.

Within four days of conception, a primitive fluid filled cavity (the primitive yolk sac) forms in the developing blastocyst; the inner cell mass lies within this cavity attached to the invading trophoblast (Hertig et al 1954; fig 1.6a). At around eight days, the inner cell mass begins to separate from its trophoblast attachment with the development of a fluid filled space, the amniotic cavity (Hertig et al 1954; fig 1.6b).

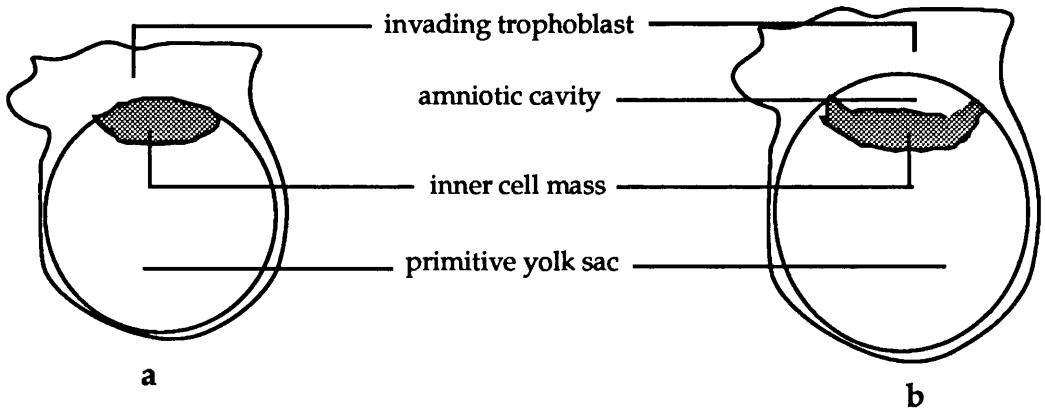

Figure 1.6 Schematic diagram to illustrate a developing human pregnancy at four days (**a**) and eight days gestation (**b**).

Whilst trophoblast at the embryonic pole invades the uterine wall to establish a vascular link with the maternal circulation, proliferation of the rest of the trophoblast causes small spaces (lacunae) to develop within the wall of the primitive yolk sac. As these lacunae enlarge they coalesce to create a new cavity outside the primitive yolk sac, the chorionic cavity or

extra-embryonic coelom (Hertig et al 1954; fig 1.7a). The chorionic cavity expands rapidly, and by the 19th to 20th day completely surrounds the embryo, which now consists of a bilaminar disc attached to its trophoblastic shell by a small connecting stalk (Jones and Brewer 1941), with the amnion on the ectodermal surface and the yolk sac on the entodermal surface (fig 1.7b).

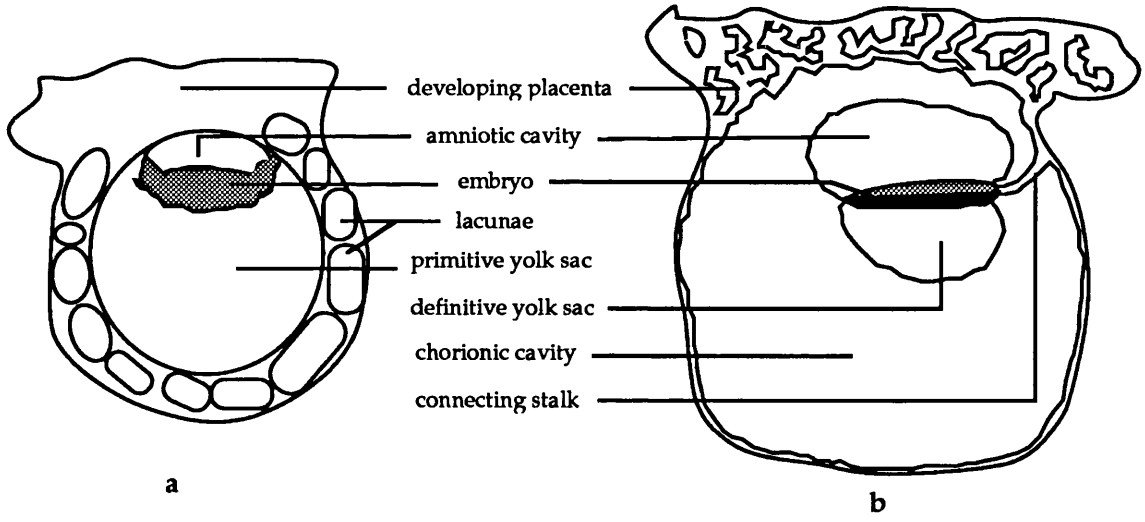

Figure 1.7 Schematic diagram illustrating a developing human pregnancy at twelve (**a**) and nineteen days gestation (**b**).

Over the next two weeks the embryonic disc undergoes rapid development and differentiation, part of which results in its cephalo-caudal and lateral folding. This draws the amnion around the developing embryo, until it is completely enshrouded by the amniotic cavity, with only the attachment to the yolk sac (vitelline duct) and the connecting stalk (umbilical cord) passing out in to the chorionic cavity (Fig 1.8; Langman 1975). Thus by the sixth week of development the fetal membranes have established their final

relationship, with the amnion inside the chorion (Fig 1.8).

During the remainder of the first trimester the amnion expands as the volume of amniotic fluid increases (Nelson 1972; Figure 1.9), until it reaches the chorion by 12-14 weeks gestation (Langman 1975). The two membranes retain this relationship for the remainder of the pregnancy, acting as one united membrane.

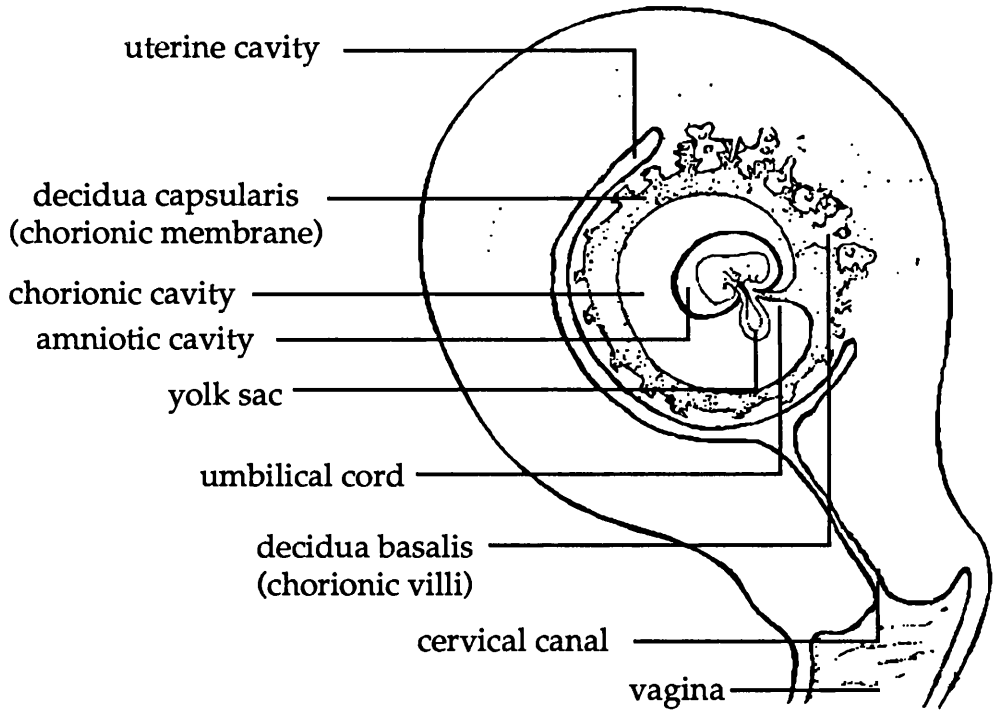

Figure 1.8 Diagram of developing pregnancy at 8 weeks gestation, to demonstrate the relationship between the amnion and chorion.

Thus amniocentesis performed in the first trimester will require the passage of a needle through two membranes to sample the small volume of amniotic fluid available (Nelson 1972) and must avoid the fetus which

occupies a relatively higher proportion of the cavity than at any other gestation. For these reasons it could be predicted that early amniocentesis will be a potentially more difficult procedure than conventional amniocentesis and may be associated with increased risk to the pregnancy. Also unique to early amniocentesis is the potential for sampling the chorionic cavity (extra-embryonic coelom) which may influence the culture success or the type of cells cultured and therefore potentially affect the karyotype.

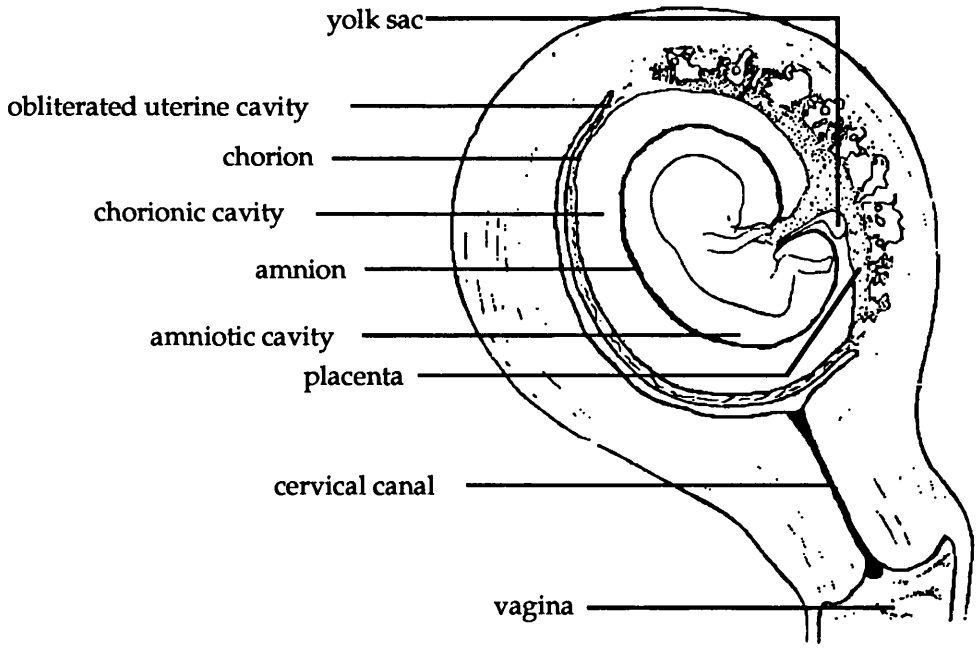

Figure 1.9 Diagram of developing pregnancy at 11 weeks gestation

Despite the predicted difficulties clinicians have attempted early amniocentesis and initial reports result from experience with attempts at increasingly early stages in pregnancy rather than a prospective evaluation of the technique. The increased resolution of modern ultrasound enables the differentiation of the separated amniotic and chorionic membranes, and coupled with increased experience of needle sampling have lead to reports of successful early amniocentesis. This section critically reviews the current literature on early amniocentesis in chronological order.

Hanson FW, Zorn EM, Tennant FR, Marianos S and Samuels S. Amniocentesis before 15 weeks gestation: Outcome, risks and technical problems. American Journal of Obstetrics and Gynaecology. 1986; 156; (6): 1524-1531.

Summary

This paper from California describes experience of amniocentesis prior to 15 weeks' gestation. Between 1979 and 1986, 11.4% (541) of amniocenteses were performed early; four procedures at 11 weeks, 36 at 12, 149 at 13 and 352 at 14 weeks' gestation. Fifteen to 35 mls amniotic fluid (mean 25 mls) was aspirated after a single needle insertion in 89% cases. Outcome was only available for 298 patients (57%) and showed 1.7% miscarried within 2 weeks of the procedure and by completion of the study 4.7% (14) had lost the baby (spontaneous abortion, stillbirth or neonatal death). There was no difference in outcome if the sample was performed transplacentally or not. However, the authors found that if pregnancies complicated by vaginal bleeding were excluded then the miscarriage rate dropped significantly (x^2=3.91; P=0.05) and

that below 35 years of age it fell to zero. They did not experience post-procedure chorioamniitis, oligohydramnios or vaginal bleeding, although there is no discussion of how these were excluded. The harvest time is difficult to interpret as the time interval chosen is not explained, being described as the time to first harvest (presumably time to subculture). There is no mention of culture failures. No cases of pseudomosaicism, confined abnormality or maternal cell contamination were reported.

Discussion

This is the first report of successful genetic amniocentesis as early as 11 weeks gestation. However the number of procedures performed at the earlier gestations are too small to derive useful information, only 4 procedures were at 11 weeks', the majority (93%) being carried out at 13 and 14 weeks' gestation. There is no discussion about how cases were selected or why they were performed early, making it impossible to establish if this was a representative sample. The sampling was successfully carried out with one needle insertion in the majority of cases (89%) but less frequently than would be expected in the second trimester, suggesting this may be a technically more difficult procedure.

Whilst there is no discussion about how the aspiration volume was chosen, the mean volume of 25 ml is large. Interestingly, they state that they removed less during early amniocentesis than conventional amniocentesis, which is surprising as most operators only remove 20 ml at conventional amniocentesis. In contradiction to reports of second trimester amniocentesis (Tabor et al 1986), the authors found transplacental sampling did not carry increased risk of pregnancy loss, although the small size of the study and incomplete follow up make this conclusion premature. The

miscarriage rate in followed-up cases was four times the rate with conventional amniocentesis (16 weeks onwards) in their unit, suggesting an early procedure may carry greater risk to the pregnancy. However, the small size and uncontrolled design of the study make it impossible to draw useful conclusions about procedure-related risk.

American Journal of Human Genetics 1987 supplement 41 and 1988 supplement 43

Summary

These two articles summarise the results presented at meetings of the American Society of Human Genetics during 1987 and 1988, and the main findings are shown in Table 1.10.

First Author	Year	Gestational Age (weeks)	Number of Patients	Sampling Success	Culture Success	Harvest Time (days)	
						mean	range
Sandstrom	1987	11 - 13	19	100 %	100 %	11	10 - 12
Garrison	1988	12 - 13	12	100 %	100 %	10	----
Goodmillow	1988	12 - 14	600	98 %	----	----	----
Arnovitz	1988	12 - 14	142	100 %	100 %	----	----
Miller	1988	11 - 15	491	99 %	100 %	----	6 - 12
Benacerraf	1988	11 - 14	108	100 %	97 %	24	----

Table 1.10 American experience of early amniocentesis, 1987-1988.

Discussion

These preliminary reports illustrated the high rate of successful sampling which experienced centres could achieve, comparable to that of conventional amniocentesis. The remarkably high culture success rates were surprising at such early gestations, as prior to this experience it had been stated that the amniotic fluid was almost acellular before 14 weeks gestation (Watchel 1969) and therefore culture would not be possible.

The wide variation in the harvest time reflects differing definitions of harvest time. Some laboratories subculture their amniotic fluid specimens and record this as the harvest time, whereas others use the time the cells are harvested for karyotyping and others the time at which a report is generated. The most useful result in clinical terms is the time it takes to obtain the report, but this is subject to variation in laboratory practices, so a more practical parameter would be the time taken from sampling to the time of culture harvest for karyotyping, which is only dependent on cell growth.

The authors concluded that early amniocentesis was a reasonable alternative to CVS (Miller 1988), that it offered exciting potential advantages for patient privacy and pregnancy management (Sandstrom 1987). Goodmillow (1988) went as far as to state that early amniocentesis offered a safe and accurate alternative to CVS. Clearly from the limited size and uncontrolled design of these studies such conclusions could not be justified. Surprisingly there was little focus on the potential complications of the procedure, Miller hypothesised that the volume of amniotic fluid removed is related to safety, and suggested that if smaller volumes of fluid were aspirated the pregnancy loss may be reduced. However, none of these early reports specifically

advised caution with the procedure, indicated potential complications peculiar to the early gestation or suggested any sort of randomised comparison with existing techniques.

Johnson A and Goodmillow L. Genetic amniocentesis at 14 weeks or less. Clinical Obstetrics and Gynaecology. 1988; 31; (2): 345-354.

Summary

Johnson and Goodmillow (1988) reviewed development of early amniocentesis by discussing both new methods of performing the technique and recent advances in cytogenetics. They clearly established the inadequacy of published and presented data to answer the question of safety, and drew attention to premature statements to the contrary. The review discussed the limited cellular content of amniotic fluid in early pregnancy and the concern over removal of such high proportions of the total amniotic fluid volume (Hanson et al 1978). The authors cited the advice of Elejalde et al (submitted for publication and cited as 1987, actually published 1990) that only 1 ml should be removed per week of gestation, as this reduced volume appeared to provide suitable material for culture. The authors drew attention to the technical differences between sampling amniotic fluid in the first and second trimesters. As in the first trimester the amniotic sac is separated from the chorionic sac by the extra-amniotic space (extra-embryonic coelom), the aspiration needle must cross the two membranes separately. The limited intra-amniotic space and the presence of the fetus increase the risk of fetal trauma compared to the same procedure performed after 14 weeks, when the extra-amniotic space is obliterated and there is "relative polyhydramnios".

Discussion

This review cited the first reference to a comparison between early amniocentesis and conventional amniocentesis (Varner et al 1987). The study compared 134 cases of early amniocentesis to age matched controls having conventional amniocentesis for the same indications. They found a statistically significant increase in the number of multiple needle insertions per successful procedure in the early amniocentesis group. There was no difference in sampling or culture failures or abnormal test results. This study, although small, further illustrates that the earlier technique is more difficult than second trimester amniocentesis and this increase is likely to be reflected by a higher number of sampling failures.

Rooney DE, MacLachlan N, Smith J, Robello MT, Loeffler FE, Beard RW, Rodeck C and Coleman DV. Early amniocentesis: a cytogenetic evaluation. British Medical Journal. 1989; 299: 25.

Summary

This was the first British report of early amniocentesis and described the authors experience of 40 cases from 8-14 weeks' gestation. All samples were obtained prior to elective termination of pregnancy, so no information of pregnancy outcome was available. Culture and cytogenetic analysis was successful in all samples taken after 12 week's gestation, but in only 68% below this gestation. In one case, culture showed a normal female karyotype at amniocentesis, but subsequent examination of the aborted material revealed a normal male karyotype, the sample was later confirmed as maternal urine. The mean time to harvest cell cultures was 1.6 days longer than their mean time to harvest cultures at 16-19 weeks gestation. They

concluded that early amniocentesis could be used, from 12 weeks gestation, as an alternative to current methods, they cautioned that the risks of the procedure were unknown and called for a randomised trial of the technique. Caution which was echoed in a subsequent letter (Meade and Grant 1989), pointing out that removing amniotic fluid in early pregnancy affects both fetal lung development and function.

Discussion

This was the first report to concentrate on the cytogenetic possibilities of earlier sampling, by evaluating successful karyotyping in relation to gestational age. The authors used this experience to conclude that 12 week's gestation was the lower limit for the procedure, which is unjustified on the basis of such a small sample size. They illustrate some of the technical difficulties of early amniocentesis by describing the erroneous sampling of maternal urine, although they state that the woman was so obese that an early procedure may have been declined in a clinical setting.

Fourth International Conference on Chorion Villus Sampling and Early Prenatal Diagnosis. Athens 1989.

Summary

The concern over false positive and to a lesser extent, false negative results encountered with CVS had encouraged operators to investigate early amniocentesis as an alternative method of diagnosing fetal karyotype and the experience of three groups were presented at the above meeting. Their findings are summarised in Table 1.11.

First Author	Year	Gestational Age (weeks)	Number of Patients	Sampling Success	Culture Success	Abnormal Result
Cuoco	1989	10 - 13	127	----	96.1%	2.4 %
Lituania	1989	11 - 15	125	99%	----	2.4 %
Stripparo	1989	12 - 15	195	----	99%	3.1 %

Table 1.11 Reports of early amniocentesis from The Fourth International Conference on Chorion Villus Sampling and Prenatal Diagnosis 1989.

Discussion

Only one group quoted their sampling success, which was high supporting the earlier American experience (Table 1.10). All three centres aspirated only 10 mls amniotic fluid for all gestations and achieved high culture success rates. Two of the groups sampled earlier than 12 weeks' gestation, which Rooney et al (1989) had advised would result in increased culture failure.

Whilst the overall failure rate was low (3.9%) the failure rate by gestation was 18% at 10 weeks, 6% at 11 weeks, 2% at 12 weeks and 0% at 13 weeks' gestation, supporting the concept that culture failure is gestation dependent (Cuoco et al 1989).

The incidence of chromosomal abnormalities at 2.4%-3.1%, is similar to that found in second trimester amniocentesis series, although as not all the authors state the indication for amniocentesis, it is impossible to determine if the groups are comparable.

Richkind et al (1989) reported their laboratory findings with early amniocentesis at the same meeting. They found the incidence of chromosomal abnormality in comparable groups of samples at 11-13 weeks was 6%, whereas at 14-16 weeks it had fallen to 3%, reinforcing the view that earlier testing will detect more chromosomal abnormalities, most of which may not survive to the second trimester

Evans MI, Drugan A, Koppitch FC, Zador IE, Sacks AJ and Sokol J. Genetic diagnosis in the first trimester: The norm for the 1990's. American Journal of Obstetrics and Gynaecology. 1989; 160; (6): 1332-1339.

Summary

The authors reviewed all 4,721 invasive prenatal diagnostic procedures performed in their unit for three years up to January 1988; 227 of which were amniocentesis performed at 10-14 weeks. The patients chose between CVS, early amniocentesis or conventional amniocentesis but were not randomised in any way. There was increasing uptake of early amniocentesis over the three years, although it never accounted for more than 7% of procedures (peaked during the last six months of the study). The majority (63%) of early amniocentesis samples were collected at 14 weeks' gestation, with only 15% of procedures carried out at 12 week's or earlier.

In all cases only one needle insertion was required, although difficulty with membrane tenting was acknowledged in 5% of samplings. The pregnancy loss following all procedures (Table 1.12) was four times greater following CVS than after early amniocentesis.

Procedure	Number	Pregnancy loss within 1 week		Pregnancy loss upto 24 weeks gestation	
		n	%	n	%
Conventional amniocentesis	3666	9	0.25 %	11	0.30 %
Early amniocentesis	227	1	0.40 %	1	0.40 %
Chorion villus sampling	1055	8	0.80 %	17	1.61 %

Table 1.12 Pregnancy loss after chorion villus sampling, conventional and early amniocentesis.

Discussion

As this is an uncontrolled non-randomised study the results must be interpreted with some caution, however the ratio of 4:1 pregnancy losses in the CVS and early amniocentesis groups respectively is an encouraging finding for early amniocentesis. Also the similar loss rate in the two amniocentesis groups, if confirmed by larger studies, would mean that the indication used for conventional amniocentesis could legitimately apply to early amniocentesis, as the balance of risks is the same. The authors concluded that the increasing proportion of patients requesting early amniocentesis reflected their growing acceptance of the procedure. However, this may just have been an effect of the operators confidence or increasing public awareness of the technique. This paper did not discuss neonatal morbidity, specifically fetal pulmonary complications, an aspect which must be addressed in a large study.

Chadefaux B, Rabier D, Dumez Y, Ouray JF and Kamoun P. Eleventh week amniocentesis for the prenatal diagnosis of metabolic diseases. Lancet. 1989; 1; (8642): 849.

Summary

In the same year Chadefaux et al reported that amniotic fluid from early pregnancy could be used to diagnose metabolic disease. They measured enzyme levels in amniotic fluid at 12 and 11 weeks gestation and were able to diagnose fetal argininosuccinuria and fetal propionic-acidaemia in two separate pregnancies, which was confirmed by examination of the abortus. Using similar techniques they were able to exclude methylmalonic-acidaemia, propionic-acidaemia and argininosuccinuria in six other at risk pregnancies.

Discussion

This demonstrated for the first time that early prenatal diagnosis of these inherited disorders of metabolism no longer depended solely on CVS.

Elejalde BR, Elejalde M, Thelen D, Trujillo C and Karman M. Prospective study of amniocentesis performed between weeks 9 and 16 of gestation: Its feasibility, risks, complications and use in early genetic prenatal diagnosis. American Journal of Medical Genetics. 1990; (35): 188-196.

Summary

The authors described the increased need for early prenatal diagnosis as a result of both recent changes in American abortion law and the parents desire for the privacy provided by early test results. Whilst CVS was

available to fulfil this early role, the high rate of pseudomosaicism (1.2%; Brambati et al 1985), false positive and negative results (Canadian Collaborative Study 1989) detracted from its acceptability in America by clinicians and parents alike.

They performed a prospective study of amniocentesis between 9 and 16 weeks gestation in 615 consecutive patients, comparing an early amniocentesis group (9-14 weeks) with a conventional amniocentesis group (15 and 16 weeks). Both groups were similar for 1) indication for procedure, 2) age at delivery, 3) parity and 4) socioeconomic group. In each case they aspirated 1 ml of amniotic fluid for every week of gestation, except at 16 weeks where 20 ml were taken. The concentration of cells (cells per ml amniotic fluid) was measured using an Elzone cell counter, which counted cells by size. After the procedure, all the patients were asked to report back if they experienced any complications and to return for a routine follow up scan 4 weeks later, only six (0.9%) did not do so.

Of the 615 procedures, 96.7% of samples were obtained at the first attempt, the remainder after two needle insertions. Six patients (0.96%) were postponed because loops of bowel, bladder or placenta obscured the needle path. The culture of samples was performed by standard methods and was successful in 99.7% cases. Seven patients (1.1%) complained of amniotic fluid leakage after the procedure, 3 at 12 weeks' and one at each of 13, 14, 15 and 16 weeks gestation respectively, whilst 18 (2.9%) complained of post-procedure abdominal cramps. There were 9 (1.46%) pregnancy losses after the procedure, 5 within the first week, 1 within two weeks, 2 within three weeks and 1 within six weeks (Table 1.13).

Gestation	Number of procedures	Spontaneous Abortions (%)
9	3	----
10	6	----
11	18	2 (11)
12	77	3 (3.9)
13	98	2 (2)
14	121	1 (0.8)
15	137	1 (0.7)
16	155	----

Table 1.13 Pregnancy loss after amniocentesis (Elejalde et al 1990).

There were seven chromosomal abnormalities (1.1%) in the whole group, composed of three male Down's syndrome (47,XY,+21), two Turner's syndrome (45,X), one balanced translocation (46, XX, t[X:2]), and one confined abnormality (47,X,-X,+3,+t(X;3)). The latter case was confined to the early amniocentesis culture and was not present at repeat amniocentesis at 20 weeks' gestation or in the aborted fetus, both of which showed Turner's syndrome (45, X). There were four pseudomosaic results, but there were no additional invasive procedures required in any of these cases. The early amniocentesis group at 12-14 weeks' gestation did not differ from the later samples (15-16 weeks) in time to obtain cytogenetic results but, before 12 weeks' gestation the reporting time was longer. There were only very small numbers in this latter group. Amniotic fluid leakages, postponed procedures, chromosomal abnormalities and miscarriages were more

common in the 9-11 weeks gestational age group.

Discussion

The authors discuss their findings in relation to previous work on the safety and diagnostic accuracy of CVS, drawing comparison with the National Institute of Child Health and Human Development study (NICHD 1989). Their post-amniocentesis vaginal bleeding rate of 0.16%, compares favourably with the 7.3% reported for CVS (NICHD 1989). The same is true of post procedure cramping, with only 2.92% complaining in this study compared to 21.9% after CVS (NICHD 1989). The total post-procedure pregnancy loss at 3.1% is similar to that found after CVS (3.0%; NICHD 1989). Despite the similar risk it is important to remember that there are major differences between the two procedures. Amniocentesis, even at early gestations takes approximately 1-2 minutes to perform, whereas transvaginal CVS takes 15 minutes or more. Chorion villus sampling is not only a more painful procedure than amniocentesis (Elejalde et al 1990) but also carries a higher risk of serious maternal infection (Barela et al 1986, Blakemore et al 1985). There is a greater chance of obtaining an inconclusive result with CVS; in this study, cytogenetic results were achieved in 99.7%, whereas for CVS in the NICHD study it was 97.8%. As a result, more patients underwent an additional invasive procedure after CVS (2.4% NICHD), than after amniocentesis (0.3%). In this study the false positive rate was low (0.3%), but included one confined abnormality [47, X, -X, +3, t(X;3)], suggesting that EA may depend to some extent on trophoblast cell lines independent of the fetal karyotype, one of the criticisms of CVS .

The major criticism of this paper is that there is no randomised control group. Although the authors divide the gestational ranges into early

amniocentesis and conventional amniocentesis this is entirely arbitrary, on the basis of when they attended for the test. The women who attended later in pregnancy may have done so because of early pregnancy problems (bleeding, pain) and thus biased the groups. The best approach would have been randomisation to a group prior to any test.

Despite the authors attempts at forming two comparable groups of amniocentesis (early and late), most of the discussion revolves around comparisons between amniocentesis and CVS. As there were no CVS patients in their study the comparisons are drawn with other published work, the danger of which is that differing techniques are used in different units, so the comparison may not be applicable.

The authors concluded that there was a significant rise in cell count at 12 weeks gestation and that this might have been the reason why the harvest time was shorter at 12 weeks gestation and above. There are two flaws in this argument; firstly, they counted all cells in the amniotic fluid, a proportion of which are dead and therefore do not contribute to cell cultures. Secondly, increase in cell number may not directly influence the speed at which the cultures grow, as more cells would increase the chance of successful culture, but the cells would still grow at a similar rate. Larger cell colonies might provide more mitoses and achieve sufficient numbers for harvesting sooner, although this remains to be confirmed.

The authors concluded that the removal of 1 ml of amniotic fluid for each week of gestation did not appear to reduce the size of the amniotic sac. This is somewhat surprising as from their pooled data on amniotic fluid volume, their amniocentesis specimen would constitute 30% of the total volume at 10

weeks, 20% at 11, 21% at 12, 18% at 13 and 11% at 14 weeks gestation. Possibly the difference in sac size was not evident because of the difficulty in accurate measurement. The decrease in amniotic fluid volume after EA is one of the unknown factors of this technique and deserves greater consideration.

Nevin J, Nevin NC, Dornan JC, Sim D and Armstrong MJ. Early amniocentesis: Experience of 222 consecutive patients, 1987-1988. Prenatal Diagnosis. 1990; 10: 79-83.

Summary

The authors present data on early amniocentesis from 9-14 week's gestation, however only 5 (2%) of the cases were performed before 12 week's gestation. The population was self selected depending on the gestation at which the patient presented. They aspirated between 2 and 17 ml of amniotic fluid, but do not justify either the wide range or the low starting volume. Sampling and culture were successful in all cases after a mean harvest time of 10 days. The chromosomal abnormality rate was high (5%), but there is no mention of whether pseudomosaicism, confined abnormality or maternal cell contamination were encountered. Their spontaneous fetal loss rate was 1.4% (Table 1.14).

Discussion

This is a review of one units' experience illustrating that early amniocentesis is a highly successful procedure. However, the uncontrolled design means the outcome figures may not be applicable to the general population. Indeed, the fetal loss rate quoted (1.4%) is lower than the background abortion rate for this gestation (2.1%, Gilmore and McNay 1985). The population included

pregnancies at high and low risk of genetic abnormality which may have significantly different risks of pregnancy loss, further confusing interpretation. The sampling volume used was a curiously wide range and probably reflects what was possible rather than proposed, as it is difficult to imagine that they planned to remove only 2 ml of amniotic fluid. This in turn reflects the difficulty of the procedure and questions the 100% sampling success. The authors conclude that their results show early amniocentesis from 9 weeks gestation can provide reliable results and offers a safe alternative to CVS, but these conclusions are premature and questionable.

Stripparo L, Buscaglia M, Longatti L, Ghisoni L, Dambrosio F, Guerneri S, Rosella F, Lituania M, Cordones M, De Biasio P, Passamonti U, Gimelli G and Cuoco C. Genetic amniocentesis: 505 cases performed before the sixteenth week of gestation. Prenatal Diagnosis. 1990; 10: 359-364.

Summary

Stripparo et al present 505 cases of EA from 11-15 weeks gestation (Table 1.14), of which 91% were at low risk of chromosomal abnormality. They achieved absolute sampling success (only 3 cases required two needle insertions) sampling 1 ml per week of gestation and high culture success (98.4%). All but 3 cases were followed up to delivery, 93.9% were livebirths, there were 11 therapeutic terminations and one neonatal death of a premature growth retarded baby. The fetal loss rate fell with advancing gestation from 14.8% at 11-12 weeks to 2.1% at 13-14 weeks to 0.9% at 15 weeks, the difference between the first two groups was significant ($P<0.001$), whilst not so between the latter two. Consequently the authors concluded that early amniocentesis between

13-15 weeks was safe.

There were 10 (1.98%) chromosomal abnormalities of which nine were autosomal trisomies and one triploidy. One sample revealed a pseudo-mosaic karyotype not confirmed by subsequent cordocentesis at 18 weeks'.

Discussion

The paper concludes that early amniocentesis from 13 weeks is a suitable alternative to conventional amniocentesis but that earlier sampling is not because of the increased fetal loss. As only 55 procedures were performed prior to 13 weeks gestation, there is not enough evidence for this qualification. Conclusions on the safety of the procedure will only be appropriate after a randomised controlled trial of sufficient magnitude to answer this question.

Penso CA, Sandstrom MM, Garber MF, Ladoulis M, Stryker JM and Benacerraf BB. Early amniocentesis: Report of 407 cases with neonatal follow-up. Obstetrics and Gynaecology 1990; 76; (6): 1032-1036.

Summary

In this paper, 407 cases of early amniocentesis from 11-14 weeks gestation were reported of which 92% were low risk cases. Successful sampling was achieved in all cases and culture in 98.3%, with a reporting time of 14-21 days. There were 15 chromosomal abnormalities, four autosomal aneuploidies, two sex chromosome aneuploidies, five mosaics and three others. The five mosaic results were composed of one maternal cell contamination, one artifactual mosaic (pseudomosaic) and one confined

abnormality not confirmed on follow up amniocentesis. All but 18 patients were followed up, resulting in 364 (93.6%) livebirths, 15 (3.6%) fetal losses and 10 (2.4%) therapeutic terminations of pregnancy.

The fetal loss rate was reviewed in relation to gestational age of sampling and contrary to the previous report (Stripparo et al 1990) no correlation was found between the two. The authors examined the neonatal outcome of their group and discovered a preterm delivery rate of 6.6%, fetal growth retardation in 6.1% and that 6.1% of neonates had pulmonary complications. The first two findings were not different to their general population rates. The latter finding was composed of respiratory distress syndrome in 1.6% neonates, all except one of whom were born prematurely, eight cases of transient tachypnoea of the newborn, two cases of apnoea secondary to congenital abnormality, two secondary to neonatal depression and one case of pneumonia. These findings are no more frequent than the authors expect from their general obstetric population. They found that the occurrence of orthopaedic deformities (scoliosis, talipes and congenital dislocation of the knees) was associated with post amniocentesis fluid leakage. Three of eight affected neonates were born to women who complained of such leakage.

Discussion

This study is the first to include neonatal follow-up and draw attention to neonatal orthopaedic deformities after early amniocentesis. They advised larger studies with the power to discover if such associations were real or not. They did not find an increase in neonatal pulmonary complications, although only symptomatic babies were examined. Ideally all babies (or a random selection) should be examined by formal lung function testing.

There was significant maternal morbidity reported in this study; 5.4% of women complained of vaginal bleeding, 2.6% of fluid leakage and 1.8% of abdominal cramps, the latter two features were more common than previously reported (Elejalde et al 1990).

The authors concluded that early amniocentesis was diagnostically accurate, only 3.4% required a further invasive procedure; 1.7% because of culture failure, 0.7% because of an ambiguous result and 1% because of subsequent raised alpha-fetoprotein. The overall post procedure fetal loss rate corrected for termination of pregnancy was 2.3%, which if sustained with larger numbers is likely to be acceptable to women at low risk of chromosomal abnormality.

First Author	Total Patients	Number of patients per gestational week							Sampling Success (%)	Culture Success (%)	Abnormal Karyotype (%)	Pseudomosaic	Maternal Cell Contamination	Confined Abnormality	Spontaneous Fetal Loss (%)	Neonatal Pulmonary Complications (%)
		9	10	11	12	13	14	15								
Nevin	222	1	2	2	26	61	130		100	100	5	---	---	---	1.4	0
Stripparo	505			13	42	158	182	110	100**	98.4	2	---	---	1	3.1	---
Penso	407			9	179	177	42		100	98.3	3.8	1	1	1	3.6	6*
Total	1134	1	2	24	247	396	354	110								

*see text
** 0.6% required two needle insertions

Table 1.14 Distribution of patients and procedures and outcome as reported by Nevin et al 1990, Stripparo et al 1990 and Penso et al 1990.

Hackett GA, Smith JH, Rebello MT, Gray CTH, Rooney DE, Beard RW, Loeffler FE and Coleman DV. Early amniocentesis at 11-14 weeks gestation for the diagnosis of fetal chromosomal abnormality - A clinical evaluation. Prenatal Diagnosis. 1991; 11: 311-315.

Summary

The authors reported that 110 women were offered early amniocentesis, four women (3.6%) had the procedure deferred because of uterine retroversion or placental position. Of the remaining 106 cases, 102 were successfully performed at the first attempt. However, in four cases sampling was suboptimal, resulting in dry taps (2), of whom one had a repeat early amniocentesis and one a CVS (after two subsequent dry taps at 14 and 15 weeks respectively) or blood stained samples (2), of which one was repeated. Culture and cytogenetic analysis was successful in all cases that reached the laboratory, with a harvest time of 8-30 days (mean 12.2 days). There were 6 (5.7%) abnormal results, two balanced translocations, two pseudomosaics and two tetraploid karyotypes. The tetraploid karyotypes were thought to result from culture artifact, both had a repeat amniocentesis confirming normal karyotypes, the pseudomosaics were identified as culture artifact, but the anxiety of one mother necessitated a repeat amniocentesis. Outcome is reported for 64 cases (60%), from which there were 62 livebirths, two fetal losses, 27 pregnancies continue and no follow up is available in 11.3%. There were two cases of amniotic fluid leakage, one at 13 weeks (one week post procedure) and the other at 24 weeks gestation, the former resulted in a normal livebirth and the latter is still ongoing, but no mention of how far progressed. There were four congenital abnormalities, one imperforate anus, one lingual heamangioma and two positional talipes equinovarus deformities.

Discussion

The authors conclude that early amniocentesis from 11 weeks' gestation is technically possible and without any additional risk over conventional amniocentesis. This conclusion is difficult to justify when technical considerations meant 3.6% women could not have the procedure in the first instance and that one in thirteen women had a repeat procedure for either sampling difficulties (8 cases) or confusing results. The follow up is incomplete and therefore interpretation of the outcome figures is impossible.

Rebello MT, Gray CTH, Rooney DE, Smith JH, Hackett GA, Loeffler FE, Horwell DH, Beard RW and Coleman DV. Cytogenetic studies of amniotic fluid taken before the 15th week of pregnancy for earlier prenatal diagnosis: A report of 114 consecutive cases. Prenatal Diagnosis. 1991; 11: 35-40.

Summary

This is a laboratory based study comparing early amniocentesis with conventional amniocentesis, by the same authors as the previous paper and incorporates the same early amniocentesis samples. They compare 114 early amniocentesis samples from 11-15 weeks' gestation with 114 maternal age-matched samples taken at 16 weeks or greater. The amniotic fluid volumes received were 4-20 ml in the early amniocentesis group and 2-31 ml in the conventional amniocentesis group. The mean harvest time was 12.2 days for early amniocentesis and 10.6 days for conventional amniocentesis. The cytogenetic results revealed eight abnormalities in the early amniocentesis group and three in the conventional amniocentesis group (Table 1.15)

Karyotype	Early amniocentesis	Conventional amniocentesis
Normal	106	108
Balanced translocation	3**	0
Trisomy 21	0	1
Pseudomosaicism	2*	1
Tetraploid	2*	0
Maternal cell contamination	1	1
Failed cultures	0	3

*Mentioned in the previous paper
**Two of these were discussed in the previous paper

Table 1.15 Cytogenetic outcome of early and conventional amniocentesis samples.

Discussion

This is the only laboratory based study reported and the only study to include a control group, providing the first opportunity for a comparison of early and late amniocentesis. As the study is laboratory based there is no explanation about clinical aspects such as, the wide variation in amniotic fluid volume which varied from seemingly inadequate to excessive, especially in the early amniocentesis group.

Abnormalities (including maternal cell contamination and pseudomosaics) were 2.6 times more frequent in the early amniocentesis group, however as already discussed the two tetraploid results were considered artifactual (Hackett et al 1991). Excluding these results there are still twice as many chromosomal abnormalities in the early group.

There were equal rates of maternal cell contamination but twice as many

pseudomosaics in the early amniocentesis group, which even if recognised as such still can cause enough anxiety to warrant repeat testing, as in the one case quoted. Such findings can therefore affect the overall safety of the technique. In the conventional amniocentesis group the pseudomosaicism consisted of only one abnormal cell (trisomy 8) in 100 cells examined, a minor degree of pseudomosaicism.

The only cultures which failed were in the conventional amniocentesis group, possibly as a result of the small amniotic fluid volume collected in some of these cases, although the authors make no comment on this. As a result of the success of the early samples they conclude that early amniocentesis from 12 weeks gestation provides suitable samples for culture and karyotype, with a similar harvest time to conventional amniocentesis and without the need for special culture techniques. Although the authors successfully cultured and karyotyped all five samples at 11 weeks' gestation, they are reluctant, on the basis of their previous findings (Rooney et al 1989) to recommend the procedure below 12 weeks gestation. This is perhaps an unjustified limitation when the literature has reported at least 126 early amniocentesis procedures at 11 weeks' gestation (see Table 1.16) with good culture success. The study on which the authors base their reluctance, only performed 7 procedures at 11 weeks and successfully cultured 5 of them (71%), whereas the literature supports early amniocentesis from at least 11 weeks gestation, possibly earlier.

First Author	Year	Number	of	patients	per	week	(failure	rate %)		Over all culture
		8	9	10	11	12	13	14	Total	success rate
Hanson	1987				4	36	149	352	541	100%
Sandstrom	1987								19	100%
Varner	1987								134	--
Garrison	1988								12	100%
Goodmillow	1988								600	98%
Arnowitz	1988								142	--
Miller	1988								491	--
Benacerraf	1988				4	60	28	16	108	--
Williamson	1988				4	7	53	133	197	98%
Resta	1988					48	273	174	495	99%
Rooney	1989	1 (40)	9 (40)	8 (25)	7 (29)	7 (0)	6 (0)	2 (0)	40	53%
Cuoco	1989			11 (18)	33 (6)	56 (2)	27 (0)		127	96%
Lituania	1989				13	35	56	12	116	--
Stripparo	1989					4	70	70	144	99%
Richkind	1989				3	6	55		64	--
Evans	1989			5 (0)	10 (0)	21 (5)	48 (4)	143	227	98%
Eljalde	1990			6	18	77	98	121	320	99%
Nevin	1990		1	2	2	26	61	130	222	100%
Stripparo	1990				13	42	158	182	395	98%
Penso	1990				9	179	177	42	407	98%
Klapp	1990		12	12	9	13	6		52	100%
Hackett	1991				5	24	42	35	106	100%
Parker	1991				2 (0)	2 (0)	10 (20)	7 (14)	21	72%
Rebello	1991				23	23	49	37	132	100%
Total		1	22	44	159	666	1366	1456	5112	

Table 1.16 Current published experience of early amniocentesis at 8-14 weeks gestation.

Early amniocentesis: summary

1. Amniocentesis is possible from 8 weeks' gestation. Culture success appears to be gestation dependent, but the practical lower limit of the procedure is still to be determined.

2. Early amniocentesis is technically more difficult than conventional amniocentesis and is associated with a higher rate of multiple needle insertions and failed sampling. Despite this, in experienced centres, sampling success is 97% or greater.

3. The harvest time for cell culture appears to be similar to second trimester amniocentesis, although varying definitions of harvest time make inter-study comparisons difficult. The most practical measure of cell harvest is time from sampling to time of harvest for karyotype, as this is dependent on cell growth and not laboratory reporting practices.

4. There is variation in the volume of amniotic fluid removed at early amniocentesis. One ml per week of gestation is quoted as a guide but it appears that a 10 ml sample gives reliable culture results, making little point in removing more.

5. Preliminary investigation of early amniocentesis indicate it is not a high risk procedure, but the small sample size and uncontrolled design of current reports mean it is not possible to establish its safety and diagnostic accuracy. A prospective randomised controlled trial in a low risk population is required. The lower gestational limit for the trial should be determined by culture success in a pilot study.

1.5 Aims of this thesis

1. To examine systematically the feasibility of true first trimester amniocentesis (before 14 weeks) and to compare the ability to obtain a sample and cytogenetic result, in relation to the conventional technique of first trimester CVS.

2. To develop a method of limiting the amniotic fluid volume removed at early amniocentesis by filtration of cells in the amniotic fluid. This will be investigated by; (i) selecting an appropriate filter material and pore size, (ii) establishing the best method for removing cells from the filter, (iii) examining the biochemical and temperature changes of the amniotic fluid after filtration, (iv) achieving successful culture and cytogenetic analysis of filtered cells and (v) determining the fetal karyotype using the method in ongoing pregnancies.

Chapter 2

Feasibility of first trimester amniocentesis

2.1 The cellular content of amniotic fluid in early pregnancy

2.2 Pilot study to determine the lowest gestational limit for amniocentesis

2.3 Technical and cytogenetic comparison of early amniocentesis and chorion villus sampling

2.4 Preliminary results on safety of early amniocentesis versus chorion villus sampling

Chapter 2. Feasibility of first trimester amniocentesis

Although there are several studies which have reported on the feasibility of obtaining amniotic fluid for cytogenetic analysis in early pregnancy they have provided conflicting results and the number of cases in which true first trimester amniocentesis was performed is very small.

This section describes three studies which aim to determine (i) the practical lower limit for early amniocentesis, (ii) to compare the technical and cytogenetic aspects of first trimester amniocentesis with CVS and (iii) provide preliminary results on pregnancy outcome following early amniocentesis and chorion villus sampling.

2.1 The cellular content of amniotic fluid in early pregnancy

Introduction

The concentration of cells in amniotic fluid has been measured from 15-24 weeks gestation in samples taken at conventional amniocentesis (Gosden and Brock 1978). There are relatively few cells at the lower end of this gestational range but the concentration increases with advancing pregnancy. The limited number of cells at 15 weeks has led some authors to suggest that "before 14 weeks the amniotic fluid is almost acellular" (Wachtel 1969). Obviously there is a cellular component before 14 weeks because cell culture from early amniocentesis is successful. However, there is limited information on the cellular content of early pregnancy (less than 15 weeks) and specifically of the live cell component.

Elejalde et al (1990) measured the total cell content of amniotic fluid from 9-16 weeks gestation using an Elzone cell counter. They established that cells were present in amniotic fluid of early pregnancy but in very low numbers. They described a significant drop in total cell number below 12 week's gestation and suggested that this as an explanation for the increased culture time they experienced in samples below 12 weeks'. However there were only 27 pregnancies studied below this stage of pregnancy and considering the small size the authors advised further investigation to confirm or refute their findings. They made no attempt to determine the proportion of live cells. The mechanical cell counter (Elzone) they used, counts cells by size and would therefore not differentiate between amniotic fluid cells and red blood cells as the sizes overlap (Bergstrom 1979). Thus, even microscopic contamination with blood would significantly increase the cell count. The best way to differentiate between red blood cells and amniotic fluid cells is by direct visualisation or by immunological markers.

The aim of this study is to establish the cellular content of amniotic fluid in the first half of pregnancy and to quantify the live cell component, in an effort to explain the apparent paradox of culture success similar to second trimester amniocentesis samples in the presence of greatly diminished cell number.

Patients and methods

Amniocentesis was performed in 125 pregnancies at 8-18 weeks gestation. A careful ultrasound scan was carried out to confirm fetal viability, establish the placental site and plan the best site for needle entry, avoiding the

placenta. After cleaning the maternal skin with alcohol based antiseptic solution, a 20 gauge spinal needle was introduced into the amniotic sac under continuous ultrasound guidance. The first 1 ml of amniotic fluid was discarded, to reduce the risk of maternal cell contamination, and the subsequent 2 mls collected for analysis.

In 78 cases the procedure was performed as a diagnostic amniocentesis to determine fetal karyotype and in 47 cases it was performed immediately before elective termination of the pregnancy. All patients gave their written consent after explanation of the procedure and the protocol was approved by the hospital ethics committee. The gestational age was determined by the last menstrual period and confirmed by the fetal crown-rump length or biparietal diameter measurement.

After collection, the amniotic fluid was agitated to ensure even distribution of cells. A 0.1ml aliquot of each sample was incubated with 0.02 ml Trypan blue at 37°C for five minutes. The solution was then examined on an improved Neubauer haemocytometer counting chamber under light microscopy. The total number of cells and the number of viable cells were counted twice for each sample and the mean result recorded. Dead cells absorb the dye and stain dark blue, whereas live cells do not, retaining a golden yellow colour (Gosden 1978).

Results

Cytogenetic analysis revealed normal karyotypes in all samples. The total number of cells increased in an exponential fashion between 8 and 18 weeks' gestation from a mean of 6,600 cells/ml at 8 weeks to a mean of 36,600

cells/ml at 18 weeks (y=987.6*10^[1.1098e^{-} 2x]; r=0.9, p<0.001: figure 2.1). The number of live cells did not change significantly over this gestational range (y = -1574.9 + 90.534x; r=0.36; figure 2.2).

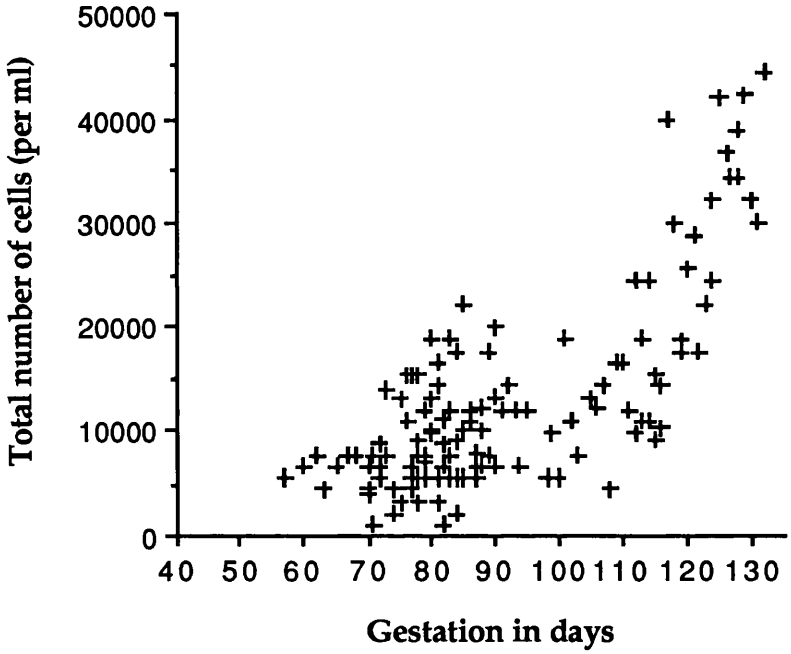

Figure 2.1 Total number of cells per ml of amniotic fluid related to gestation.

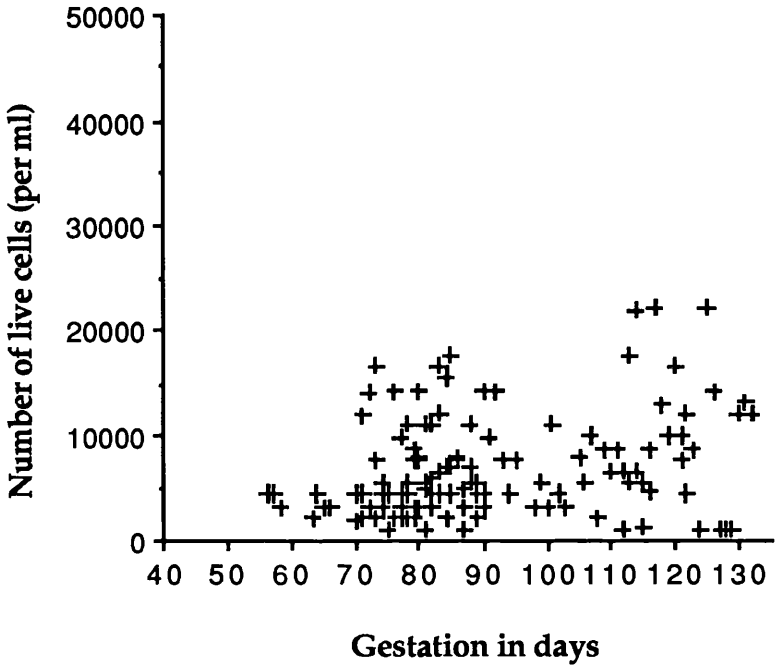

Figure 2.2 The number of live cells per ml amniotic fluid related to gestation.

The relative proportion of live cells decreased with advancing pregnancy from 61% at 8 weeks to 19% at 18 weeks gestation (figure 2.3).

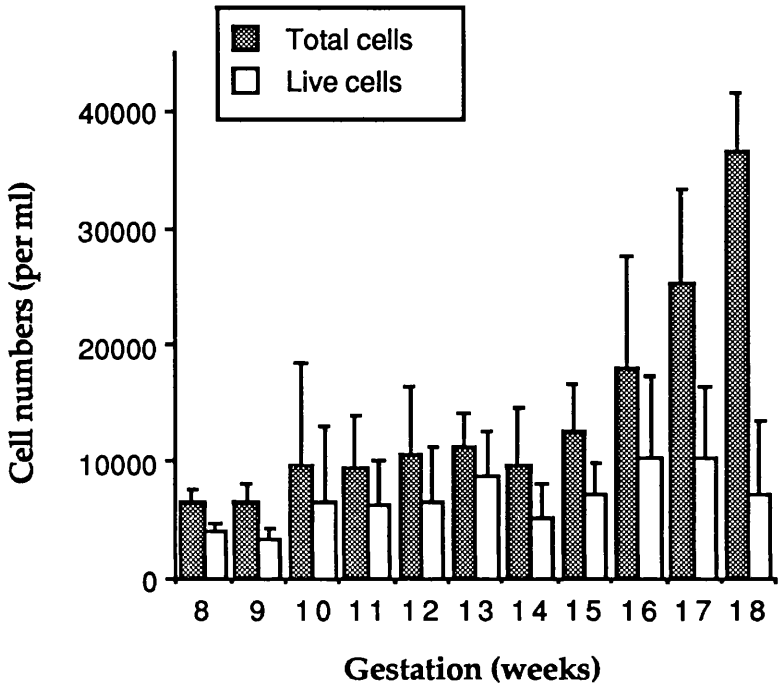

Figure 2.3 Mean total and live cell count per ml amniotic fluid in relation to gestation.

Discussion

Although the total number of cells in amniotic fluid during the first trimester of pregnancy is less than that of the second trimester, the number of live cells is relatively similar at the two stages. This finding offers an explanation for the apparent paradox that cell culture and karyotyping is possible in the first trimester of pregnancy despite the fact that the total

number of cells is low.

The increase with gestation in the number of dead cells may be a consequence of fetal urination and the addition of cells from the fetal urinary tract, diluting the viable cells which come from the fetal periderm (Bergstrom 1979). The fetus is believed to start passing urine from approximately 14 weeks gestation (Lind 1972). However, this hypothesis requires direct evidence by studying the origin of the different cell populations in amniotic fluid.

2.2 Pilot study to determine the lowest gestational limit for early amniocentesis.

Introduction

The aim of the current study is to establish if cell culture from amniotic fluid in the first trimester is as successful as the second trimester as predicted from section 2.1, and to define the lower practical limit for early amniocentesis.

Patients and methods

Ultrasound guided amniocentesis was performed using a 20 gauge spinal needle in 56 women immediately prior to elective termination of pregnancy at 60-93 (mean 76) days gestation. The gestational age from menstrual dates was confirmed by ultrasound measurement of the fetal crown-rump length (CRL) which was 25-75 mm (mean 46mm). All patients gave their written consent and the study was approved by the hospital ethics committee. After

needle placement 1 ml amniotic fluid was aspirated and discarded, to avoid the risk of maternal cell contamination. Subsequently 10 ml was aspirated and transferred in a sterile container to the cytogenetics laboratory for culture.

Culture technique

The amniotic fluid sample was identified and logged in the culture record. Using aseptic techniques and working in a laminar flow cabinet, the fluid was divided equally into three sterile, labelled, culture tubes. These were centrifuged at 1300 rpm for 10 minutes, returned to the safety cabinet where the supernatant was poured off and the cell pellet resuspended in 2 ml of culture medium (Ham's F10, Hepes buffered [25 mMol with L-glutamine] and supplemented with 5% fetal calf serum, 2% Ultroser G and antibiotics [penicillin and streptomycin]). Unlike second trimester amniocentesis, the cell pellet was not always visible at early gestations. The culture tubes were sealed, lain flat and incubated at 37°C.

After five days the culture tubes were examined under an inverted microscope for signs of cell growth (adherent cells and colony formation). On day seven if growth was evident the culture medium was changed with fresh medium, if there was no cell growth then only half the medium was changed. Thereafter the culture medium was changed on alternate days. Cultures which did not grow were maintained for three weeks before being discarded as failed.

Thymidine synchronisation

Synchronised harvesting with thymidine was used to increase the number of cells in mitosis at the time of harvest. The day prior to harvest the culture

medium was changed with fresh medium containing 0.1 ml thymidine solution (15gm/l distilled water). Eight to ten hours later this medium was poured off, the tube rinsed with fresh medium twice, new fresh medium added and the sample incubated at 37°C overnight. The subsequent morning 0.05 ml of 0.01% colchicine was added for 30 - 120 minutes and the culture examined for rounded cells, at which point it was harvested.

Harvest using the suspension method

The contents of the culture tube were transferred to a labelled centrifuge tube, containing versene (0.2gm/L isotonically buffered saline). The original tube was rinsed with versene and then incubated at 37°C for ten minutes with versene in it to lift off the remaining cells. No other agents were used to lift off the cultured cells. To confirm all the cells have lifted off, the tube was examined under the inverted microscope and the remaining versene poured into the centrifuge tube. The sample was centrifuged at 1000 rpm for 10 minutes, supernatant poured off and 1% tri-sodium citrate added and incubated at 37°C for 30 minutes. Once again the tube was centrifuged and the supernatant poured off. A pipette full of prepared fixative (3:1 analar methanol : gliacial acetic acid, chilled in a freezer) was added and the tube agitated, manually. The sample was centrifuged once again, the supernatant poured off and the remainder transferred on to slides. The slides were aged by incubating overnight at 60°C and then examined by G-Banding.

G-Banding

The slide was dipped in 1% trypsin solution (in phosphate buffered saline) for between 30 seconds and 2 minutes, then washed with water. It was stained with a Giemsa solution (1 part stain to 10 parts buffer solution, pH 6.5) for 4 minutes, washed with water and air dried. Karyotype analysis was

performed from at least two independent cultures on at least 15 mitoses from each.

Results

Amniotic fluid was obtained in all cases and successful culture and karyotyping was achieved in 47 of the 56 (84%) samples. Culture was successful in all 39 cases where the fetal crown-rump length (CRL) was >37mm, but in only 8 of the 17 (47%) with CRL ≤37mm. A CRL of 37 mm is equivalent to a gestation of 71 days (figure 5.1).

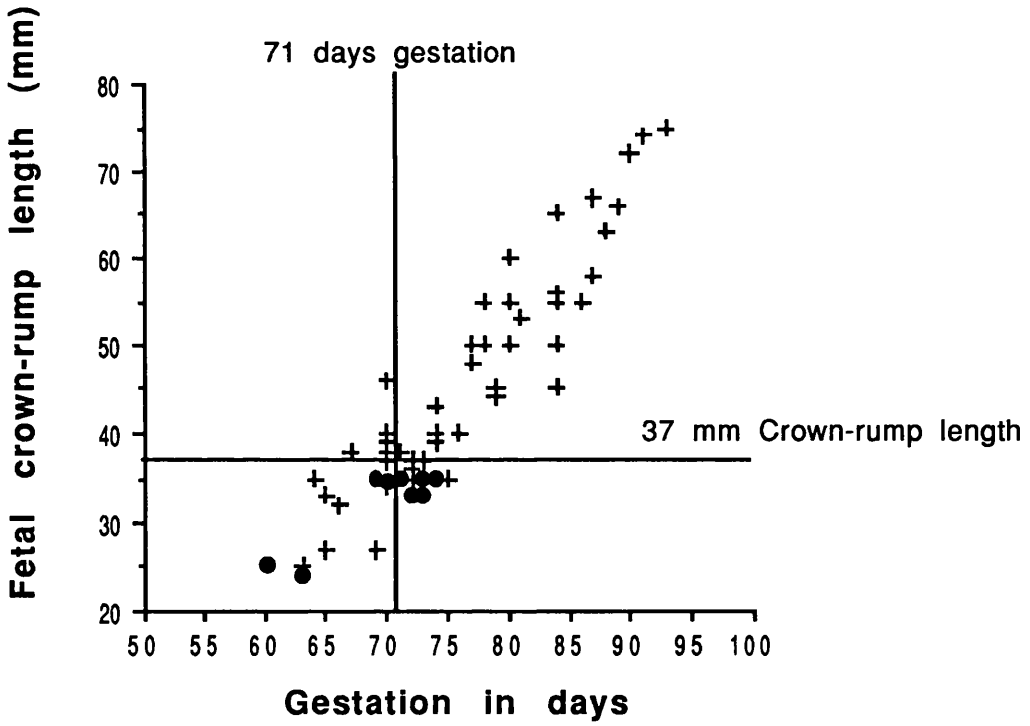

Figure 2.4 Culture success (+) and failure (•) in relation to fetal crown-rump length and gestational age of pregnancy.

The fetal karyotype was 46, XY in 23 cases, 46, XX in 23 cases and 47, XY, +21 in one case. The mean interval between sampling and harvest was 12.1 days (range 5-18 days; SD=2.7).

Discussion

Whilst this pilot study confirms that ultrasound guided amniocentesis and culture of amniotic fluid is possible from eight weeks gestation, it establishes that success of culture in very early pregnancy is inadequate for practical use. However, if a minimum fetal CRL of 38 mm is accepted as the lower limit for the procedure the success rate is as high as second trimester amniocentesis (Tabor et al 1986). The gestational equivalent of this CRL does not clearly divide the successful cultures from the failures (figure 5.1) and is therefore not as useful a cut off.

The cultures were prepared using the same methods as second trimester amniocentesis samples, illustrating that early amniocentesis will not entail additional work in the laboratory. In contrast, sample preparation of CVS specimens requires more time and expertise than amniocentesis (Association of Clinical Cytogeneticists Working Party, 1987). Thus, the findings of this preliminary study suggest that early amniocentesis may provide the same advantages as CVS (successful cytogenetic analysis in early pregnancy), without the additional laboratory workload and therefore cost.

2.3 Technical and cytogenetic comparison of early amniocentesis and chorion villus sampling.

Introduction

The aim of this study is to compare the technical aspects of early amniocentesis with the technical aspects of the currently available technique for prenatal diagnosis of fetal karyotype in early pregnancy, CVS. It is also aimed to illustrate any differences in cytogenetic outcome between the two procedures. The study is a prospective randomised trial of two techniques and the reason for the design is discussed below.

Choice of control group

The other techniques to which early amniocentesis could be compared are second trimester amniocentesis and CVS. To compare with conventional amniocentesis the randomisation would have to be performed in the first trimester before either treatment has occurred. The subject group would have their amniocentesis shortly after randomisation whilst the control group wait until 16 week's gestation for their amniocentesis. This delay will inevitably lead to some disappointment and possible drop out (Muggah et al 1987). Such non compliant patients might request the alternative test or seek help elsewhere. This is likely to be disproportionately weighted toward the second trimester amniocentesis group and may introduce bias between the groups. This is exactly what happened in the American amniocentesis versus CVS trial (Rhoads et al 1989).

A second reason why the comparison with conventional amniocentesis is

not the most suitable choice is *because* they are performed at different gestations. Currently patients request CVS because they wish an early test, the alternative of amniocentesis is already available but they value the earlier diagnosis offered with CVS. So, even if there are aspects of second trimester amniocentesis that are better than early amniocentesis these patients may still choose the early technique. It is therefore more relevant to compare early amniocentesis to the only other available early technique, CVS. Also randomisation can be carried out at the same time for both groups, so drop outs should be equally divided between the two.

It could be argued that technical aspects aside, CVS still offers advantages over early amniocentesis in that rapid results can be obtained using direct preparation of villi. However, there is sufficient concern that direct preparations result in an increased risk of erroneous results, so most laboratories rely on culture for their definitive result (MRC 1991). This takes 2-3 weeks, similar to amniocentesis, thereby negating the potential benefit of CVS direct preparations.

Gestational range for the trial

The pilot study (section 2.3) illustrated absolute culture success when the fetal crown-rump length was >37 mm. This establishes the lower limit for amniocentesis; there is no upper limit as it can be performed for the remainder of pregnancy. Sixteen weeks gestation is the lower limit that most clinicians currently use, although some occasionally carry out the test at 15 or 14 weeks' gestation. Thus the upper limit for this study should be 13 weeks and 6 days gestation, to separate this study from conventional amniocentesis, and the lower limit, a fetal CRL of 38 mm (10 weeks' and 2 days gestation).

Chorion villus sampling can be performed as early as 6 week's gestation (Brambati et al 1991) but the questioned safety in very early pregnancy has now limited its use to above 10 weeks' gestation (Firth et al 1991). There is no upper limit to the procedure as placental biopsy can be performed until the end of the pregnancy, like amniocentesis. Consequently the same gestational range is applicable to CVS also.

Choice of technique

Previous comparisons of CVS and second trimester amniocentesis have been criticised for two main reasons. Firstly, such studies were performed when CVS was a relatively new technique and differences may have reflected the learning curve for the newer procedure. Secondly, as there are many different methods for performing CVS (section 1.4.1), the comparison is between amniocentesis performed in a uniform manner and various techniques for CVS. As these factors contribute to the success of obtaining a sample and the post-procedure loss incurred such study design introduces potential bias between the groups.

Currently the majority of CVS procedures are performed by transabdominal needle insertion with ultrasound guidance (Smidt-Jenson 1984). Consequently, it is now possible to compare transabdominal CVS (performed with a single 20 gauge needle) with transabdominal early amniocentesis performed (with a single 20 gauge needle) by operators experienced in both techniques; overcoming some of the criticisms of previous studies.

Randomisation

The method of randomisation must respect the tremendous emotional burden parents feel when undertaking a test that carries the risk of

miscarrying a normal fetus. It is insensitive and arguably unethical to inflict a strict randomisation protocol on all who enter the study, the result would be low patient acceptability and a high drop out rate, as has been the experience of other authors who attempted such a study (Muggah et al 1987). A better option is to offer couples the choice between randomisation and making their own decision. Counselling given before they make their decision must be non directive and state that aim of the trial is to compare technical and cytogenetic aspects of the two procedures. Those who accept randomisation will be offered two identical opaque envelopes, one of which contains a card with amniocentesis written on it, the other, an identical card with CVS written on it. The mother and father, individually or together, select one of the envelopes and the choice is made. The parents who choose by randomisation are called the "envelope choice" group and the parents who choose themselves are called the "patient choice" group. The data from the groups will be collected separately and compared for dependent variables. If there is no difference between the two groups the data may be pooled. Whilst at first, this appears statistically incorrect and seems more appropriate that only the randomised data be used, it is important to appreciate that the choice between randomisation and not may produce some selection bias. Perhaps patients who allowed randomisation were from a lower socioeconomic group, possibly without the confidence to challenge the doctors. This is a powerful reason to vindicate the pooling of such data, but one must remain aware of this pooling as it may be influential in some of the findings. As regards the technical aspects of the two sampling methods and the cytogenetic outcome, I believe it is unlikely to be influential. This approach is a modified randomised consent design, which has been considered appropriate in situations where it is difficult to make patients adhere to strict randomisation (Pocock 1983).

Patients and methods

Women requesting fetal karyotyping at 10-14 weeks gestation (minimum fetal CRL=38 mm) because of advanced maternal age, anxiety or family history of chromosomal abnormality (without parental translocation) are suitable for entry into the trial. The exclusion criteria are the following:

- Missed abortion
- Multiple pregnancy
- Heavy vaginal bleeding
- Major fetal abnormality
- Multiple fibroids, making either early amniocentesis or CVS impossible
- Intrauterine contraceptive device in situ
- Operator chooses procedure, because alternative is considered too difficult

Data is collected prospectively at the time of randomisation, procedure and follow up.

Both CVS and early amniocentesis are performed by transabdominal insertion of a 20 gauge spinal needle into the uterus under continuous ultrasound guidance. For CVS the needle is directed into the placenta without puncturing the amniotic sac. Once in position the trocar is removed and a 20 ml syringe containing 1 ml of wash medium (100 ml RPMI 1640, Hepes buffered, 5000 units penicillin and 10,000 units heparin) is attached to the hub of the needle. Suction is applied by fully withdrawing the plunger of the syringe with an aspiration handle (Cameco Ltd., Sweden) and the needle is guided in and out of the placenta for 10 seconds. Villi are aspirated up the needle into the wash medium. The needle is then removed and the contents of the syringe squirted into a petri dish containing wash medium. It

is examined under a dissecting microscope, the villi separated out and transported to the laboratory in transport medium (100 ml RPMI 1640, Hepes buffered, 5000 units penicillin, 10,000 units and 20 ml, 5% fetal calf serum).

On arrival in the laboratory the chorionic villi are cleaned under a dissecting microscope, to remove any blood or decidual contamination. They are macerated with a number 22 scalpel blade to expose the mesenchymal core, picked up with a pipette and transferred to three culture tubes. They are spread evenly across the surface of the tubes, which are then inverted to drain off the excess transport medium and the tubes left to dry for 1-2 hours. Culture medium (Hams F10 medium supplemented with 2% Ultroser G and 5% fetal calf serum) is added and the tubes sealed and carefully lain flat and incubated at 37°C. Cultures are examined in the same way as the amniocentesis samples and harvested by the suspension method after synchronisation with a thymidine block (section 2.2).

For early amniocentesis a 20 gauge spinal needle is directed into the amniotic sac without puncturing the placenta. As the chorionic and amniotic sacs are separated in the first trimester of pregnancy, great care must be taken to ensure the needle has penetrated the inner (amniotic) sac. The trocar is then removed and 1 ml amniotic fluid aspirated and discarded, to reduce the risk of maternal cell contamination. Subsequently 10 ml amniotic fluid are withdrawn and transported to the laboratory in a sterile container. The amniotic fluid is prepared as previously described (section 2.2).

Results

A total of 650 women entered the study, of whom 326 had CVS and 324 had early amniocentesis. In the CVS group the procedure was randomly allocated by envelope in 164 cases and chosen by the patient in 162, for early amniocentesis, 154 patients were randomly allocated and the patient chose in 170 instances. All procedures were performed for low risk indications as described above. There was no significant difference in maternal age within or between the groups (Table 2.1).

Group	Choice	Mean age (years)	Range	(SD)	t	t
EA	Patient	38	23 - 46	(3)	0.29 NS	0.542 NS
	Envelope	38	25 - 45	(3)		
CVS	Patient	37	23 - 48	(4)	-0.083 NS	
	Envelope	38	22 - 44	(4)		

Table 2.1 Mean and range of maternal age within early amniocentesis (EA) and CVS groups, showing no significant difference (Student's t test; P>0.05) in maternal age if procedure was chosen by patient or randomly allocated by envelope.

The mean gestation for early amniocentesis was 10.9 weeks and for CVS was 11.2 weeks. The gestation was not normally distributed, so comparison of data between the groups was performed using non parametric analysis (Mann-Whitney U test) and showed no significant difference within or between the two groups (Table 2.2).

Group	Choice	Mean Gestation (weeks)	Range (SD) (weeks)	p	p
EA	Patient	11	10 - 13 (1)	U=13845.5 NS	U=-14057.9 NS
	Envelope	11	10 - 13 (1)		
CVS	Patient	11	10 - 13 (1)	U=16311.0 NS	
	Envelope	11	10 - 13 (1)		

Table 2.2 Gestation of sampling in early amniocentesis (EA) and CVS groups, showing no significant difference (Mann-Whitney U test; NS) in sampling gestation, if patient chose the procedure or it was randomly allocated by envelope selection.

Amniotic fluid was successfully obtained at the first attempt in 320 cases (98.8%) and after two needle insertions in four cases (1.2%). CVS provided sufficient material (>5 mg wet weight) for cytogenetic analysis in 323 out of 326 cases (99%), 314 (97.2%) at the first attempt and in nine cases (2.8%) after two needle insertions. In three cases, samples considered adequate by the operator were subsequently confirmed as decidual tissue only.

The median time of needle insertion was 50 seconds for CVS (range 20 - 240; SEM= 6.7) and 45 seconds for early amniocentesis (range 45-300; SEM=7.0), which was not significantly different (Mann-Whitney U, P=0.49).

Cell culture and chromosomal analysis were successful in 318 of 324 cases (98.1%) of early amniocentesis (Figure 2.5). Culture failure occurred in three out of 75 cases (4%) at 10 weeks' gestation, two of 137 (1.5%) at 11 weeks' and one of 69 (1.4%) at 12 weeks' gestation. One had a repeat early amniocentesis, three a CVS, one a cordocentesis and one declined further investigation.

Among this group four produced normal results and one yielded a double aneuploidy with trisomy 21 and a maternally inherited marker chromosome (48, XY, +21, Mar).

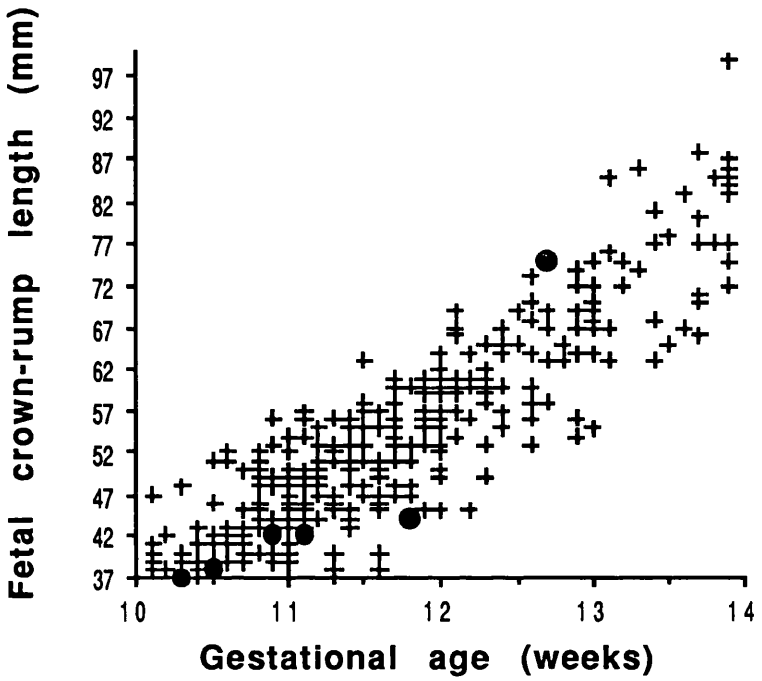

Figure 2.5 Culture success (+) and failure (•) in 324 early amniocenteses from 10 weeks gestation.

In the CVS group, culture failure occurred in two of the 323 (0.6%) cases where a suitable sample was obtained. Both patients declined the offer of repeat testing, although one subsequently changed her mind and had a cordocentesis at 20 weeks' gestation. In three cases where the sample consisted of decidua only, subsequent early amniocentesis revealed normal results.

Karyotype	Amniocentesis	CVS	Total
46,XX	150	143	293
46,XY	160	162	322
Trisomy 21	4	4	8
Trisomy 18	2	3	5
Trisomy 13	_	2	2
Trisomy 22	_	1	1
47,XXY	1	1	2
47,XY,+marker	1	-	1
Confirmed mosaics	_	5*	5
Failed	6	5**	11
Total	324	326	650

* See Table 2.4
** In 3 of these only decidual tissue was obtained

Table 2.3 Karyotype result in 324 early amniocentesis and 326 chorion villus samples.

The cytogenetic results were normal in 615 out of 639 cases (96.2%; Table 2.3) with the ratio of female to male karyotype at 1 : 1.1. Autosomal aneuploidy was the most common abnormality and of these trisomies dominated. There were no mosaic, pseudomosaic or maternally contaminated results in the early amniocentesis group, whilst there were five (0.8%) such problems in the CVS group (Table 2.4). Three of these were true mosaic results as confirmed by culture of fetal blood or skin after termination of pregnancy

(two autosomal trisomies and one 47, XXX). In one CVS sample all cells were trisomic for chromosome 2, a condition known to be associated with confined placental abnormality, but if present in the fetus is incompatible with intrauterine life. Since the fetus was developing normally (as demonstrated by serial ultrasound scans) and genetic counselling was so reassuring the parents elected to continue the pregnancy without further invasive tests. Delivery of a normal female infant resulted.

Case	Culture tube number			Outcome	Interpretation
	1	2	3		
1	7% 46,XX 93%47,XX,+8	10% 46,XX 90% 47,XX,+8	100% 47 XX,+8	Fetal blood confirmed 96% 46,XX 4% 47,XX,+8	Apparently true mosaic
2	90% 46,XX 10% 47,XX,+21	90% 46,XX 10% 47,XX,+21	———	Amniocentesis culture 93% 46,XX 7% 47,XX,+21 Fetal Skin Confirmed 70% 46,XX 30% 47,XX,+21	Apparently true maosiac
3	15% 46,XX 85% 47,XX X	23% 46,XX 77% 47,XXX	50% 46,XX 50%47,XXX	Fetal skin confirmed 93% 46,XX 7% 47,XXX	Apparently true mosaic
4	43% 46,XY 57% 46,XX	19% 46,XY 81% 46,XX	7% 46,XY 93% 46,XX	Fetal blood confirmed 46 XY Normal male infant delivered	Maternal cell contamination
5	100% 47,XX,+2	100% 47,XX,+2	100% 47,XX,+2	After counselling declined further test. Normal fetal development on ultrasound scan.	Confined placental mosaic

Table 2.4 Cytogenetic results in five chorion villus samples which presented diagnostic difficulties.

The median interval between sampling and achieving cytogenetic results for early amniocentesis was 12 days (range 8 - 22) and 11 days (range 6 - 20) for CVS (figure 2.6a and 2.6b). These times were not significantly different (Mann-Whitney U test; p=0.49).

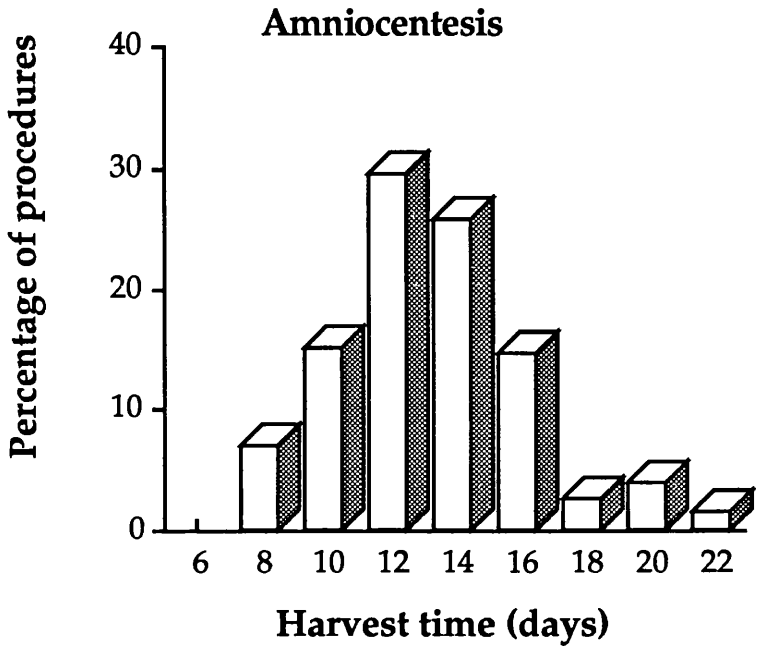

Figure 2.6a Distribution of harvest time in early amniocentesis samples.

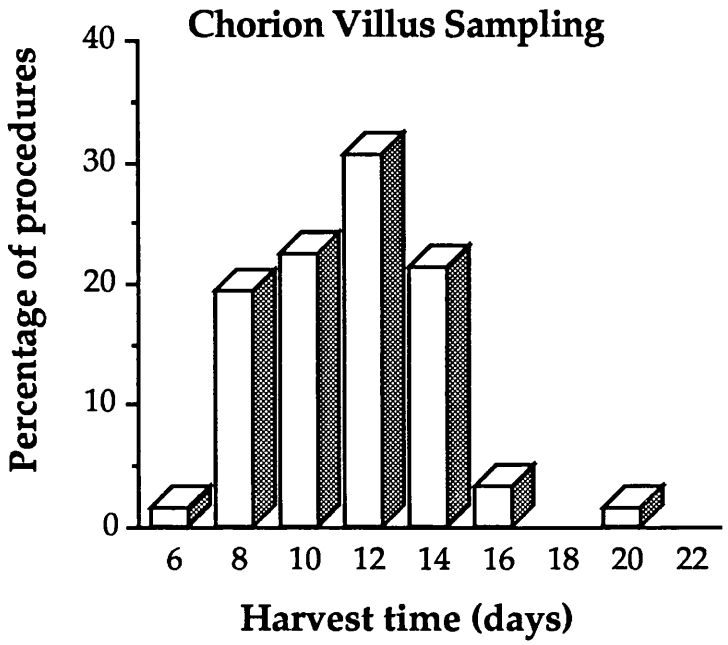

Figure 2.6b Distribution of harvest time for chorion villus samples.

Discussion

This study clearly shows that early amniocentesis is a suitable alternative to CVS for first trimester fetal karyotyping in that samples can be obtained at the same gestations and culture relied upon.

The initial concerns about the ability to sample amniotic fluid (section 1.4.2; page 64) in the first trimester appear to be unfounded, in that a sample was obtained in all cases in this study. However the procedure is undoubtably more demanding than conventional amniocentesis, because the small target size reduces the room for sampling error. In the second trimester the volume of amniotic fluid increases so fast (Nelson 1972) that relative "polyhydramnios" exists, so access is easy and the chance of fetal damage low. Thus some operators still perform conventional amniocentesis as a blind

procedure and achieve high success rates. This would not be possible in the first trimester, because the small target demands exact needle placement, to avoid maternal or fetal damage. This study has shown that in experienced hands with ultrasound guidance a sample can always be obtained. In four cases two needle insertions were required because a dry tap was obtained. This means the needle was within the uterine cavity but no sample could be aspirated. I think this is caused by the amniotic membrane being sucked against the needle and blocking it. Usually the needle is in the extra-amniotic space and swift advancement will puncture the amnion and allow sample collection. However in these four cases this was not not possible, so a new point of entry was used, successfully. The unsupported amnion in the first trimester can be extremely difficult to puncture as it "floats" away from the needle or tents when pressed. The greatest success will be obtained by a swift insertion perpendicular to the amnion surface, as the needle can bounce off if the angle of approach is too wide. In the second trimester the amnion is held taught against the chorion so such problems are rare. However even in the first trimester the amnion is held taught over the chorionic plate and this may aid successful puncture, but would require passing the needle through the placenta. In this study the placenta was deliberately avoided to reduce the risk of pregnancy loss, perhaps this is counter-productive to sampling success; only further study will demonstrate which method is best.

Accurate needle placement is also essential in CVS, the needle must be in the subchorionic area to reduce the risk of decidual contamination and obtain a suitable weight of material. As placental and uterine position is variable this sometimes results in very demanding procedures. The approach is particularly difficult if the uterus is anteverted and the placenta on the

anterior uterine wall. These technical constraints are demonstrated by the higher number of two needle insertions required in the CVS group (2.8% versus 1.2%), and by the case of maternal cell contamination from decidual tissue. As a consequence of the experience required, CVS remains limited to the few centres with suitably trained clinicians, whereas amniocentesis is widely available. For Obstetricians experienced in conventional amniocentesis, early amniocentesis will be an easier technique to learn than CVS, but the procedure must be performed with synchronous ultrasound guidance and after sufficient practice on termination samples. If it is adopted without these provisions then one could expect the sampling failure rate, and possibly the pregnancy loss and fetal damage to be unnecessarily increased.

The high rates of 96% and 98% for successful sampling at the first attempt of CVS and early amniocentesis, respectively, compare favourably with the 69% rate for CVS and 94% rate for second trimester amniocentesis reported in the European trial (MRC 1991). Furthermore, in this study the two procedures are performed in essentially the same way, transabdominal approach with a 20 gauge needle. So final data on fetal loss rates are likely to reflect the inherent risk of sampling the different tissues, rather than the heterogeneous nature of the sampling technique (transabdominal or transvaginal approach, cannula or biopsy forceps, single or double needle), which at least in part may have contributed to the findings of the Canadian, European and Danish randomised trials (Canadian Collaborative CVS-Amniocentesis group 1989, MRC 1991, Smidt-Jensen et al 1991).

In this and other studies published to date, there have been no new problems in the culture of cells from early amniotic fluid over those of second

trimester amniocentesis. It is therefore, appropriate to apply the extensive knowledge gained from over 20 years experience with traditional amniocentesis to this new technique. Initial apprehension that the small number of cells in early amniocentesis samples might enable an abnormal cell line to constitute a higher proportion of the total and represent an artificially high level of pseudomosaicism, fortunately appears to be unfounded. However, the true impact of pseudomosaicism on the cytogenetic accuracy of early amniocentesis will only be determined when larger numbers have been studied.

Successful cytogenetic results were obtained in 98% of cases for both early amniocentesis and CVS; insufficient tissue sampling in three and culture failure in two cases of CVS balanced the six culture failures in the early amniocentesis group. However the high level of culture success with early amniocentesis is only achieved when the fetal CRL is greater than 37 mm. In the majority of previous reports on early amniocentesis, the failure rates were similar to the present study (section 1.5). The 21% failure rate in the study of Rooney et al (1989) can be attributed to the high proportion of cases sampled at less than 10 weeks' gestation.

The interval between sampling and obtaining cytogenetic results is similar for the two techniques. Although with CVS much faster results can be obtained by direct preparation, the consequence of such a policy would be an unacceptably high false positive rate. The alternative of performing direct preparation and culture doubles the laboratory workload (Association of Clinical Cytogeneticists Working Party, 1987) and therefore the cost, which is of critical importance for a test aimed at 5% of the pregnant population. Furthermore, to obtain sufficient material for both direct preparation and

culture, either a larger gauge needle or repeated insertions are required. In the present study, a single 20 gauge needle insertion was chosen because it is the least traumatic method, and therefore expected to be the technique with the lowest procedure-related risk.

This study has demonstrated that early amniocentesis is a viable alternative to CVS for fetal karyotyping, in terms of successful sampling, cytogenetic results and speed with which results can be obtained. Indeed, early amniocentesis has the advantage over CVS that the processing of samples requires less experienced laboratory staff, can be performed in batches and is less labour intensive.

2.4 Preliminary results on the safety of early amniocentesis versus chorion villus sampling.

The following outcome data can only be considered a guide rather than definitive results, as the sample size is too small to draw final conclusions about the comparative safety of the two procedures. The outcome for all 650 cases undergoing first trimester fetal karyotyping is shown in Table 2.5.

	Early amniocentesis n=324 (%)	Chorion villus sampling n=326 (%)
LB	298 (92.0)	306 (93.8)
NND	1 (0.3)	-
IUD/SA	17 (5.2)	4 (1.2)
TOP	8 (2.5)	16* (5.0)

*in two cases TOP was because of anencephaly and spina bifida respectively

Table 2.5 Outcome of 650 first trimester procedures for fetal karyotype, illustrating number of livebirths (LB), neonatal deaths (NND), intrauterine deaths/spontaneous abortions (IUD/SA) and termination of pregnancy (TOP) for early amniocentesis and chorion villus sampling.

These data illustrate a similar livebirth rate for the two procedures, however there appear to be more spontaneous fetal losses (IUD/SA) in the early amniocentesis group (5.2% versus 1.2%). Whilst the number of fetal losses are small, especially in the CVS group, the difference may illustrate a trend

toward a higher complication rate with early amniocentesis.

Case	Procedure	Gestation of procedure	Outcome	Gestation of fetal loss
1	EA	12.7	IUD	13
2	EA	12.6	IUD	14
3	EA	11.3	IUD*	12
4	EA	10.9	IUD	29
5	EA	13.9	IUD	31
6	EA	11.1	IUD	26
7	EA	11.6	IUD	13
8	EA	11.3	SA	13
9	EA	12.6	SA	16
10	EA	11.7	SA	14
11	EA	11.4	SA	13
12	EA	11.4	SA	13
13	EA	10.7	SA	12
14	EA	12.3	SA	13
15	EA	10.6	SA	13
16	EA	12.4	SA	17
17	EA	11.0	SA	13
18	CVS	10.6	IUD	40
19	CVS	10.7	SA*	11
20	CVS	11.0	SA	14
21	CVS	10.4	SA	16
22	EA	12.4	NND	28

*Chorioamniitis

Table 2.6 Gestational age at which early amniocentesis (EA) and chorion villus sampling (CVS) was performed and at which fetal loss by spontaneous abortion (SA), intrauterine death (IUD) or neonatal death (NND) occurred.

The fetal losses occurred a mean of 31 days (range 1-127 days) after early amniocentesis and 85 days (range 2-275 days) after CVS. The mean gestation

of the procedure which resulted in fetal loss was 11.7 weeks (range 10.6-13.9 weeks) for early amniocentesis and 10.6 weeks (range 10.4-11.0 weeks) for CVS. Infection was identified as the cause in one case for each group (table 2.6; cases 3 and 19). There was one neonatal death in the early amniocentesis group (table 2.6; case 22), which occurred at 28 weeks gestation after emergency caesarean section for antepartum haemorrhage and one of the intrauterine deaths in the CVS group occurred at term (table 2.6; case 18).

The fetal loss rates are an unexpected finding, as the preliminary results from uncontrolled trials (section 1.4.2) suggest that early amniocentesis is as safe as late amniocentesis, which has been shown to be safer than CVS (MRC European trial 1991). Thus a higher fetal loss rate would be expected following CVS. Perhaps the reason for this paradox is explained by the design of this trial, which compared the two procedures being performed in the same way by experienced operators, rather than a variety of techniques performed in different centres by operators of varying skill, as in the MRC European trial (1991). This trial was designed to show the inherent risk of sampling the two tissues (amniotic fluid versus chorionic villi) and if this trend is confirmed on larger numbers it will indicate that sampling chorionic villi is the safer procedure. However other factors, such as cytogenetic accuracy, fetal morbidity (Firth et al 1991), cost and laboratory expertise (Association of Clinical Cytogeneticists Working Party 1987) will have to be considered before adopting one procedure in preference to the other. These will only be established with the result of a large prospective randomised trial of early amniocentesis and CVS.

Feasibility of early amniocentesis: summary

1. The number of cells in amniotic fluid increases with advancing gestation, but the proportion of live cells is greater in early pregnancy. As a result, the number of live cells at 10-14 weeks' gestation is similar to the number at 15-18 weeks' gestation.

2. There is an exponential increase in the number of dead cells in amniotic fluid from 14 weeks gestation onwards. This may be due to release of dead cells from the urinary tract of the fetus, as the fetus starts to pass urine. Only by characterising cells by their site of origin will this become apparent.

3. The pilot study performed on pregnancies prior elective termination demonstrates that amniocentesis beyond a fetal CRL of 37 mm is likely to be as successful as second trimester amniocentesis. Before this time the culture failure rate is unacceptably high.

4. To establish the technical aspects of early amniocentesis it is most relevant to compare it to the only other early prenatal test CVS. The gestational range for comparison considered useful is 10-14 weeks'.

5. In a randomised comparison of similar groups a sample was successfully obtained at the first attempt in 98.8% cases for early amniocentesis and in 97.2% cases for CVS; cell culture and chromosomal analysis was successful in 98.1% and 99.4% cases respectively.

6. Two needle insertions were required more frequently with CVS (2.8% cases) than with early amniocentesis (1.2% cases). There was no significant

difference between the tests for the time the needle was in the uterus (CVS 50 seconds, early amniocentesis 45 seconds; p=0.49).

7. Cytogenetic results revealed a normal male and normal female in 52% and 48% cases respectively. Abnormal karyotype was diagnosed in 3.8% cases, the commonest abnormality was autosomal aneuploidy.

8. There were no mosaic, pseudomosaic or maternal cell contaminations in the early amniocentesis group, whereas there was one confined placental mosaic, one maternal cell contamination and three true mosaics in the CVS group. If this disparity remains when larger numbers are studied it will represent a major advantage of early amniocentesis over CVS.

9. Preliminary results on pregnancy outcome suggest that the livebirth rate for early amniocentesis is similar to that of CVS (92% versus 93.8%), but that early amniocentesis carries a higher fetal loss rate (5.2% versus 1.2%). This is yet to be confirmed by a larger study.

Chapter 3

Reducing the volume of amniotic fluid removed at early amniocentesis

Chapter 3. Reducing the volume of amniotic fluid removed at early amniocentesis

3.1 Introduction

Ultrasound measurement of amniotic fluid volume is difficult and relies upon mathematical presumptions about the shape of the intra-amniotic contour. However, direct measurements have been made after hysterotomy (Nelson 1972). On the basis of these data the volume of amniotic fluid removed for successful early amniocentesis (11 mls) could constitute as much as 76% of the total (Table 3.1).

Gestation (weeks)	Number of cases	Mean volume (ml)	SD	Proportion removed at early amniocentesis (%)
8	5	14.5	5.9	76
9	2	8.2	---	---
10	11	29.6	12.4	37
11	10	45.4	13.9	24
12	27	73.9	35.9	15
13	17	89.9	20.9	12
14	16	111.3	43.4	10
15	18	143.7	75.7	15*
16	22	207.2	92.3	10*
17	19	233.9	104.4	9*
18	10	258.0	97.4	8*
19	3	333.3	13.3	6*
20	3	365.3	87.8	6*

*At second trimester amniocentesis 21 mls is aspirated

Table 3.1 Amniotic fluid volume during the first half of pregnancy (Nelson 1972), illustrating the proportion removed, if 11 ml were aspirated at amniocentesis.

The effect of this reduction in amniotic fluid volume is seen most dramatically with ultrasound during early amniocentesis; before the procedure there is plentiful fluid for the fetus to move freely within (Fig 3.0, upper photograph) whilst after it the membrane may collapse enshrouding the fetus (Fig 3.0 lower photograph).

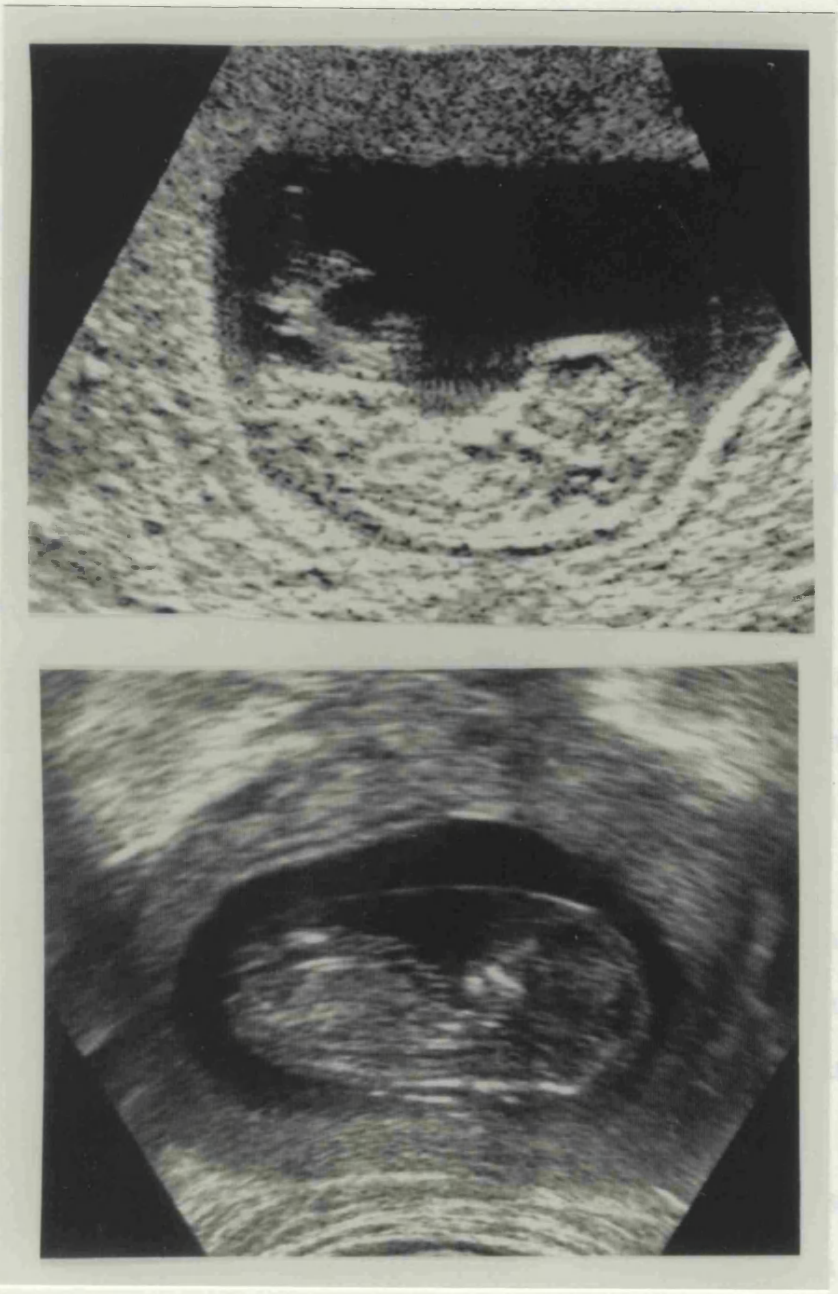

Figure 3.0 Photograph of an ultrasound scan of an eleven week fetus prior to amniocentesis, illustrating the volume of amniotic fluid (upper photograph) and after amniocentesis showing the collapsed amniotic membrane around the fetus (lower photograph).

Sometimes the deflated sac is seen as a flaccid membrane undulating between the intra and extra-amniotic compartments in response to movement of the ultrasound probe.

The effect of this change in volume on the developing fetus is less clear but experience with conventional amniocentesis and animal experiments may provide a clue. Conventional amniocentesis is performed when the fetal lung is in the canalicular period of development (17-26 weeks; Jobe 1984), which is characterised by the first appearance of potential gas exchange surfaces (Boyden 1974). These are the acinae and constitute all the respiratory structures distal to the terminal bronchiolus (Jobe 1984). During this phase there is repeated branching of the terminal airway in preparation for terminal sac development at 26 weeks (Hislop and Reid 1974). If an insult occurred during this phase which affected the distal branching then the area for gas exchange would be reduced and if sufficiently severe may result in pulmonary hypoplasia and neonatal death. Indeed this has been demonstrated in cases of severe oligohydramnios (Potter 1946, Perlman et al 1976), as well as with prolonged amniotic fluid leakage (Alcorn et al 1977).

The significant increase in unexplained pulmonary complications in the newborn of pregnancies where conventional amniocentesis has been performed suggests that even an isolated insult may impair fetal pulmonary development (MRC 1978, Tabor et al 1986). Thankfully most of these complications are mild and short-lived in the neonate, but there is no data available about possible longterm sequelae as no follow up studies through childhood and into adult life have been completed. Perhaps minor developmental complications could result in pulmonary deficit in later life, with increased risk of diseases like bronchitis, emphysema or chest

infections.

In the animal model of Cynomolgus monkeys, amniocentesis has been demonstrated to cause a reduction in the number and size of alveoli in the newborn (Hislop et al 1984). This reduction was significantly greater ($P<0.01$; Hislop et al 1984) when the amniocentesis was performed early in the pregnancy (equivalent to 14-17 human weeks), suggesting that the susceptibility to pulmonary complications is gestation dependent. Perhaps the larger proportion of fluid removed in early pregnancy produces relative oligohydramnios; which is a known cause of fetal pulmonary complications (Wigglesworth and Desai 1982). Against this theory is that Hislop et al showed a similar effect if the membranes were punctured but no fluid withdrawn (four cases). However, the authors did not confirm whether the amniotic fluid volume was maintained after the procedure, as this insult may have caused chronic fluid leakage (difficult to detect in a monkey model) and therefore produced the same effect as in the amniocentesis group. Whatever the answer, these experiments suggest that the fetus may be more vulnerable during early amniocentesis when the fetal lung is in the pseudoglandular stage of development (7-17 weeks; Jobe 1984).

Despite this evidence, it is still uncertain as to whether the pulmonary complications are due to a decrease in amniotic fluid pressure or a direct effect of amniotic fluid volume reduction. Recently, the situation has been made clearer by experiments on the fetal lamb which demonstrate that mimicking the pressure reduction of oligohydramnios, without amniotic fluid removal, does not result in fetal pulmonary complications (Fisk et al 1992), underlining the importance of maintaining the amniotic fluid volume.

At present, therefore there is sufficient concern about the possible consequences of removing large amounts of amniotic fluid in early pregnancy that every attempt should be made to reduce this volume to a minimum. Any method of prenatal diagnosis which capitalises on the cytogenetic success of early amniocentesis without removing a large volume of amniotic fluid may represent an advantage.

This chapter describes the development of a system which filters the amniotic fluid, retrieving cells whilst returning the majority of the fluid to the amniotic sac. The system has been established as a result of a series of studies to: (i) discover the most suitable filter material and pore size, (ii) demonstrate the best method of cell retrieval, (iii) ensure sterility of the system, (iv) determine biochemical changes after filtration, (v) ascertain the minimum filtration volume required for a clinically useful result and (vi) discover the temperature effects of filtration.

As preliminary outcome data has suggested that early amniocentesis may result in a higher fetal loss rate than CVS the search for a safer method of sampling amniotic fluid cells becomes more relevant than ever.

3.2 Amnifiltration studies

The following investigations, unless otherwise stated, were performed on pregnancies at 10-14 weeks gestation prior to elective termination, under general anaesthesia. In all cases amniocentesis was performed through a 20 gauge spinal needle using a freehand technique with continuous ultrasound

guidance. All women gave their written consent and the study was approved by the hospitals ethics committees. Cells obtained were cultured in the method previously described (section 2.2) and the number of clones (actively dividing group of >50 cells) in a 10 ml culture tube were counted manually using an inverted stereo microscope.

Filter type and pore size

Filters with pore sizes from 0.22 μm, 0.45 μm, 0.8 μm and 1.2 μm were tested for their ability to trap amniotic fluid cells. However, initial attempts at aspirating amniotic fluid through the smaller filters (0.22 μm and 0.45 μm pore size) proved so difficult that their further evaluation was abandoned. Thus two filters pore sizes and two materials were investigated; 0.8 μm cellulose acetate, 1.2 μm cellulose acetate and 1.2 μm polyamide filters (Sartorious GmbH, Goettingen, Germany).

Methods

Fifteen patients were subdivided into three groups of five according to the material and pore size of the filter used; group 1, polyamide 1.2 μm, group 2, cellulose acetate 1.2 μm and group 3, cellulose acetate 0.8 μm. Three samples were collected from each patient as follows: The tip of the spinal needle was introduced into the amniotic cavity and 10 mls amniotic fluid were aspirated into a syringe (Figure 3.1; sample A). Subsequently, a filter was connected between the hub of the needle and a new syringe and a further 10 mls aspirated (Figure 3.1; sample B). The filter was then removed and flushed

with 10 mls of culture medium (Figure 3.1; sample C). All samples were cultured for eight days and the number of clones generated counted.

Results

The 0.8 μm cellulose acetate filter (group 3) was found to be the most efficient filter (Figure 3.2), because it trapped all the cells (no growth in sample B) and allowed their release when flushed (high number of clones in sample C). In contrast, the 1.2 μm cellulose acetate filter (group 2) allowed a large number of cells through (many clones in sample B). Although the 1.2 μm polyamide filter (group 1) was good at trapping cells (no clones in sample B), it did not allow their release when flushed (minimal clones in sample C).

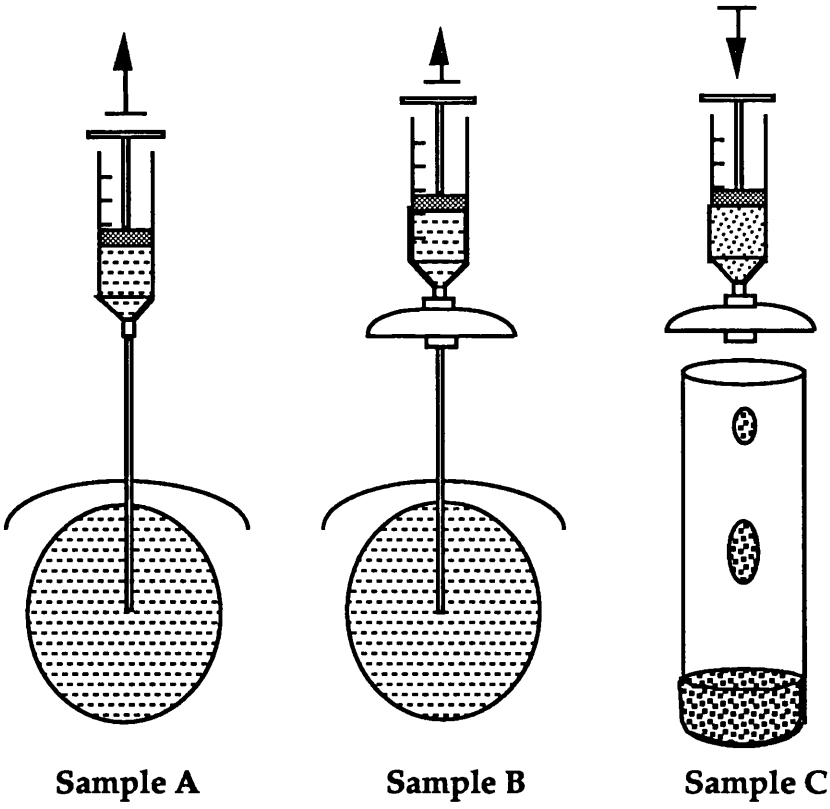

Figure 3.1 Diagrammatic illustration of types of sample used in studies discussed in section 3.1.

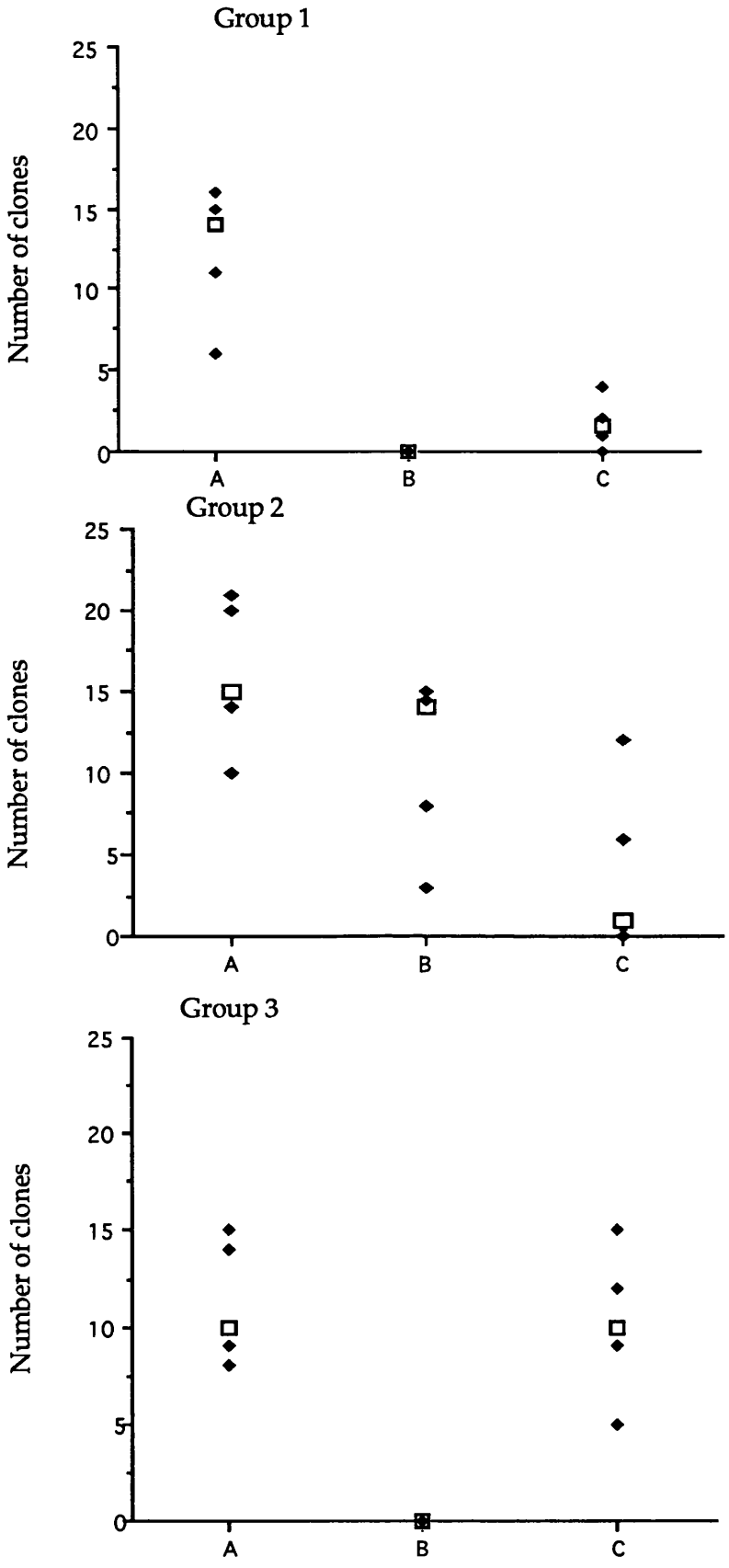

Figure 3.2 Number of clones per culture tube (◆) in samples A, B and C (section 3.1) using three different filter types: Group 1, polyamide, 1.2 µm pore size; Group 2, cellulose acetate, 1.2 µm pore size; Group 3, cellulose acetate 0.8 µm pore size. Median (□) shown for each group.

Cell retrieval from the filter surface

Whilst it is possible to maintain cell cultures on the surface of the filter membrane (Sartorious GmbH, Goettingen, Germany), it is impossible to assess cell growth and count clones under light microscopy as the filter is opaque. Thus cells must be separated from the filter and a maximum number of viable undamaged cells be regained. Two methods were considered suitable; (i) agitation of the filter membrane in medium and (ii) flushing the cells off with medium forced across the filter surface in the opposite direction to the aspiration.

Methods

(i) Agitation method

A special filter holder which allowed removal of the filtration membrane was used to filter 10 ml amniotic fluid from 5 pregnancies. After the procedure the membrane was transferred to a sterile universal container of culture medium (RPMI 1640 medium buffered with 25 mmol Hepes buffer with L-glutamine and supplemented with 20% fetal calf serum, penicillin, streptomycin and nystatin). This container was agitated on a rotomixer (Hook and Tucker Instruments Ltd, Croydon, England) at mid speed for 2 minutes. Subsequently the filter was removed and lain face down in a culture flask containing growing medium (Ham's F10 with 25 mmol Hepes buffer with L-glutamine, supplemented with 5% fetal calf serum and 1% Ultroser G and ampicillin). The original transport medium was cultured as described previously (section 2.2). The number of clones were counted after 8 days culture.

(ii) Reverse flush method

Amniocentesis was performed on ten pregnancies and 10 ml of fluid were filtered through a 0.8 μm cellulose acetate filter in each case. Subsequently 15 ml of culture medium was flushed back across the filter and collected in aliquots of 5 ml into three separate culture tubes (Figure 3.1; sample C). These were cultured for 8 days (section 2.2) and the number of clones counted.

Results

(i) Agitation method

The culture flasks containing the filters showed no growth, whilst the agitation medium grew 1-3 clones in all cases (mean=2.2).

(ii) Reverse flush method

A total of 63 clones grew in culture, of which the majority (70%) were in the first flush and 91% were contained in the first two flushes (Table 3.2).

	Sample										
	1	2	3	4	5	6	7	8	9	10	Total (%)
First flush	5	12	6	4	2	5	2	3	2	3	44 (70)
Second flush	0	8	1	0	0	0	0	0	1	3	13 (21)
Third flush	0	2	1	0	0	1	2	0	0	0	6 (9)

Table 3.2 Number of clones after 8 days culture in first, second and third flush of culture medium.

Sterility of the filtration system

A process which includes the extracorporeal circulation of amniotic fluid must be sterile or it will risk introducing infection, possibly causing chorioamniitis, congenital infection or pregnancy loss.

Methods

The filtration system, amnifilter, (Figure 3.3) is assembled from the following commercially available sterile parts, (i) a disposable filter (Sartorious GmbH, Goettingen, Germany), (ii) two three way taps (Vygon UK Ltd, Cirencester, England), (iii) bypass "T" tubing 12 cm long with an internal diameter of 0.74 mm (Medex Medical Inc, Rossendale, England), (iv) a male to male luer lock connector (Vygon UK Ltd) and (v) a 10 ml syringe. The tip of the needle is guided into the amniotic cavity, the trocar removed and the amnifilter connected to the hub of the needle. Amniotic fluid is aspirated up the bypass tubing, until all the air in the tubing is drawn into the syringe. The three way taps are then turned to direct fluid across the filter and the amniotic fluid is aspirated into the 10 ml syringe after the dead space air. By turning the three way taps the filtered fluid is redirected along the bypass tubing and returned to the amniotic cavity. This process (amnifiltration) may be repeated as many times as necessary.

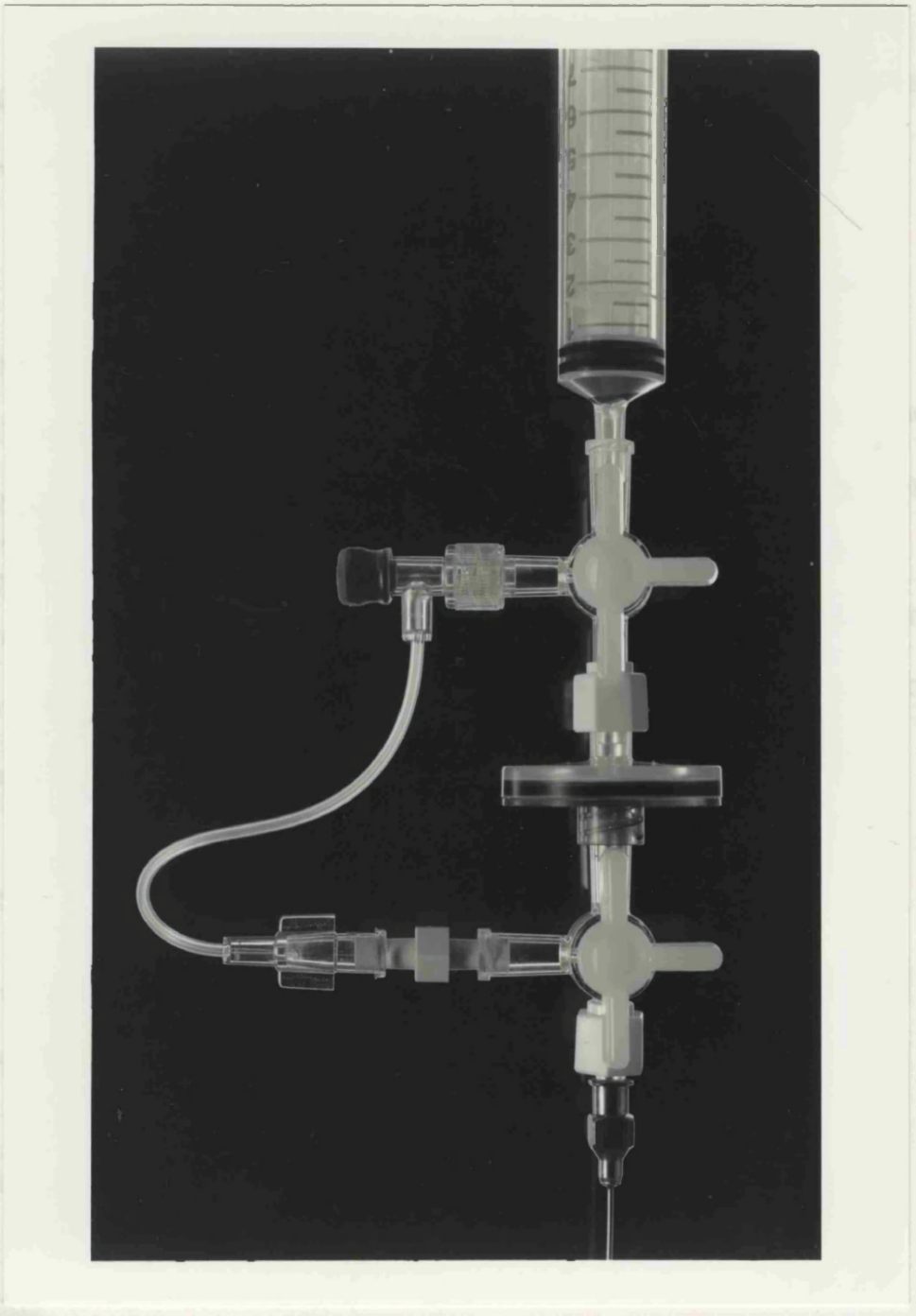

Figure 3.3 The amnifiltration system.

In 50 pregnancies amniocentesis was performed and 2 mls of fluid aspirated (Figure 3.1; sample A). Subsequently, the amnifiltration system was attached to the hub of the needle and 10 mls of amniotic fluid were aspirated into the syringe. The needle was withdrawn from the maternal abdomen and the fluid injected into a sterile tube (sample B). The amniotic fluid samples, A and B, were divided into aliquots:

(i) 10 µl were cultured on a blood agar plate at 37°C and examined at 24 and 48 hours;

(ii) 3 mls were incubated in brain heart infusion broth at 37°C for 48 hours then subcultured onto blood agar and incubated at 37°C in an atmosphere of 7% CO_2 for a further 48 hours, being examined at 24 and 48 hours; and

(iii) 1 ml was inoculated into human embryo lung fibroblasts, incubated at 37°C on roller drums and the culture maintained for four weeks being examined on alternate days for viral growth.

Results

There were no bacterial or virological agents found in any of the pre or post-filtration samples.

Biochemical changes of amnifiltration

Methods

In 20 pregnancies amniotic fluid (2 mls) was obtained before (Figure 3.1; sample A) and after attachment of a cellulose acetate 0.8 µm filter (Figure 3.1; sample B). Urea, electrolytes, protein, calcium, phosphate, creatinine and

urate were measured in each sample (Technicon SRA 2000, Technicon instruments corporation, New York, USA).

Results

There was no significant difference in the biochemical constitution of the amniotic fluid before and after filtration (Table 3.3).

		Pre Filtration			Post Filtration			
		Mean	Range	SD	Mean	Range	SD	t*
Na	(mmol/l)	137	134-141	2	137	133-141	2	1.07
K	(mmol/l)	4.0	3.7-4.4	0.2	4.0	3.4-4.4	0.2	-0.24
Urea	(mmol/l)	3.4	2.6-4.3	0.5	3.4	2.6-4.3	0.5	-0.47
Cr	(µmol/l)	64	44-82	12	64	41-86	13	0.15
Ca	(mmol/l)	1.59	0.89-2.28	0.37	1.59	0.86-2.25	0.36	-0.56
PO4	(mmol/l)	1.2	0.6-1.9	0.4	1.2	0.6-1.8	0.4	0.44
Bilirubin	(mmol/l)	0.6	0.0-2.0	0.7	0.8	0.0-2.0	0.8	-1.0
Urate	(mmol/l)	0.14	0.05-0.21	0.05	0.14	0.06-0.21	0.05	-0.82

* None are significant

Table 3.3 Mean, range and standard deviation (SD) of biochemical parameters before and after amnifiltration.

Minimum filtration volume required

The aim of this study was to establish the minimum recirculation volume needed to produce at least the same number of clones in culture as produced by a standard 10 ml amniocentesis sample, to prevent unnecessarily long filtration procedures.

Methods

In 50 pregnancies 10 mls of amniotic fluid was aspirated (Figure 3.1; sample A) followed in each case by amnifiltration with varying recirculation volumes; 10 mls in eight cases, 20 mls in ten cases, 25 mls in ten cases, 30 mls in ten cases, 35 mls in six cases and 40 mls in six cases. The number of clones produced after eight days culture was recorded for both the amniocentesis and the amnifiltration sample. For each patient the difference in the number of clones between amniocentesis and amnifiltration was calculated (Δ value).

Results

When only 10 mls of amniotic fluid was recirculated the number of clones in culture was significantly less than in the amniocentesis control ($t=2.26$; $P<0.05$). However, when the recirculation volume was 20-40 mls there was no significant difference between the number of clones generated from amnifiltration or the amniocentesis control ($t= 0.78$; $P= 0.46$; Figure 3.4).

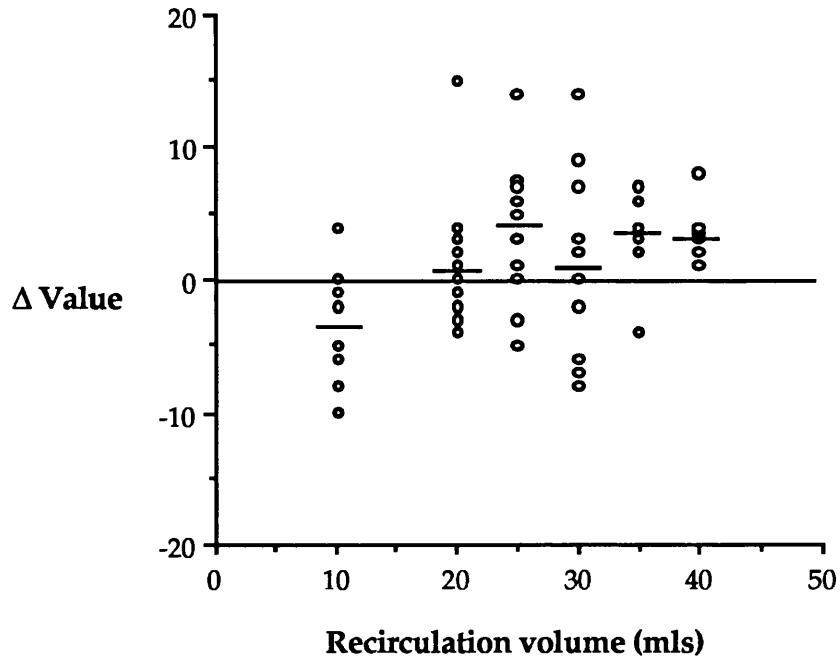

Figure 3.4 Graph showing the relationship of recirculation volume and the difference in (Δ) the number of clones generated after amniocentesis and after amnifiltration. Positive values indicate that there were more clones in the amnifiltration sample than in the amniocentesis sample and a negative value, vice versa. Median (-) shown for all samples.

Amniotic fluid temperature change

The circulation of amniotic fluid around the amnifilter can be expected to change its temperature as there is no insulation around the system. The aim of this study is to determine the extent of any temperature change so induced.

Methods

In ten patients a 20 gauge needle was directed (under ultrasound control)

into the amniotic sac, the trocar was removed and a sterile, fine copper wire T-type thermocouple was introduced into the amniotic cavity. The needle was withdrawn along the thermocouple wire, leaving the wire in place (Figure 3.5), thus enabling continuous recording of the amniotic fluid temperature on the attached electronic thermometer (51 k/J thermometer, Fluke Ltd, Illinois, USA). The electronic thermometer was precalibrated to 0.1°C over the range 35-38°C, using a precision mercury in glass thermometer (MRC Hypothermia unit, Hammersmith Hospital, London). Amnifiltration was then performed as described above (section 3.3) and three circulations of 8 mls amniotic fluid were carried out. The stable temperature was recorded on each occasion after returning the amniotic fluid to the uterus. Subsequently the thermocouple wire was gently withdrawn until it was in the peritoneal cavity, as heralded by a temperature drop and the significant length of wire removed. The peritoneal temperature was recorded as the core temperature. Lastly the room temperature was recorded with the same instrument.

Results

The mean core temperature was 36.5 °C (range 35.8-37.0; SD=0.3) and the mean amniotic fluid temperature prior to filtration was 36.8 °C (range 36.2-37.1; SD=0.3). The amniotic fluid temperature fell successively with each filtration circuit to a mean of 36.5°C after 8 mls, 36.3°C after 16 mls and to 36.2°C after 24 mls, a fall of 0.6°C (figure 3.6). The mean room temperature was 22.9°C (range 22.1-24.7; SD=0.9).

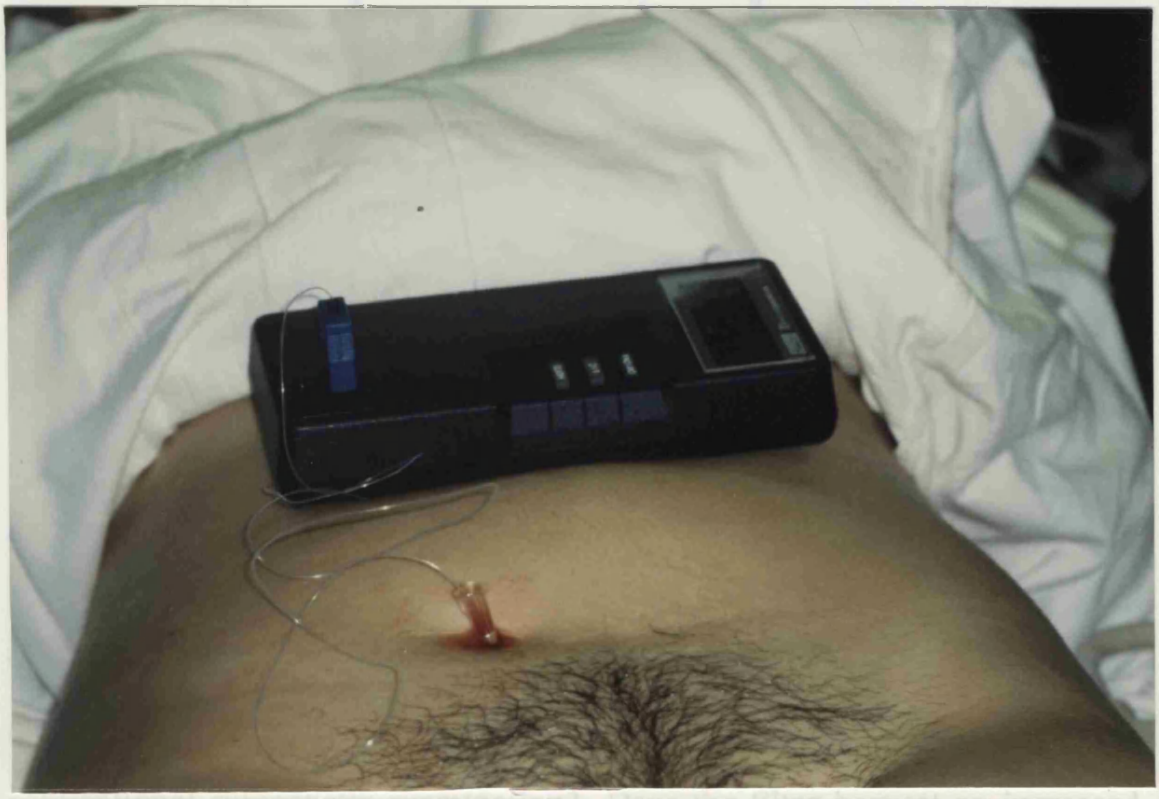

Figure 3.5 Thermocouple wire in place in to record amniotic fluid temperature.

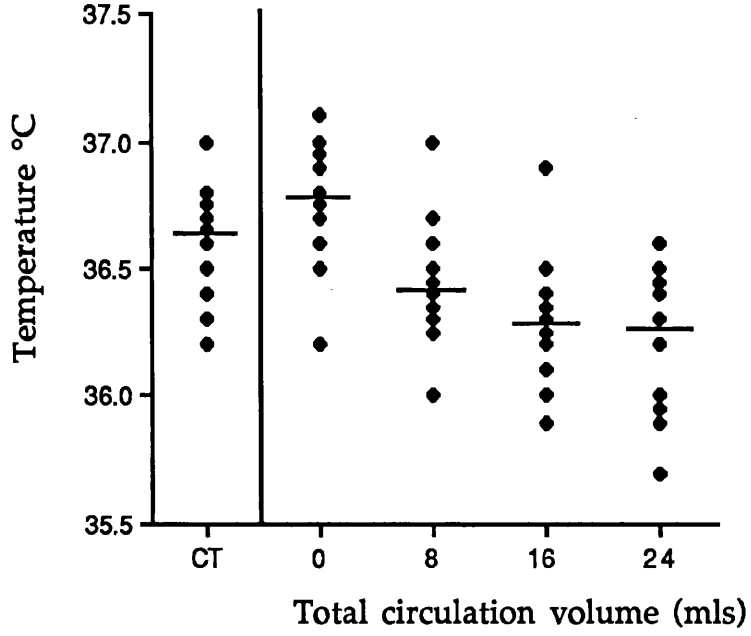

Figure 3.6 Amniotic fluid temperature (°C) in relation to increasing circulation volume through amnifilter. CT= patients core temperature. Median (-) shown for all samples.

Cytogenetic outcome of amnifiltration

The studies presented in section 3.1 illustrate that cells can be effectively trapped and retrieved using the amnifilter, but are the cells suitable for conventional cytogenetic examination? Does the filter harvest an increased number of cells from the fluid, such that cultures will be quicker and more reliable? The aim of this study is to demonstrate that filtered cells can be karyotyped using conventional cytogenetic methods.

Methods

In 38 pregnancies, prior to elective termination at 10-14 weeks' gestation, amnifiltration was performed using a 0.8μm pore size cellulose acetate filter (section 3.1.2) and a total of 24 mls amniotic fluid was filtered in three 8 ml passes (section 3.1.6). The system was then disassembled and 10 mls of culture medium (section 2.2) flushed across the filter in the opposite direction to the initial aspiration. The flush was transferred to the laboratory in a sterile container, where it was cultured and karyotyped using the same method as early amniocentesis (section 2.2).

Results

All cultures were successful and the karyotype normal male (46, XY) in 20 cases and normal female (46, XX) in 18 cases. The mean harvest time of cultures was 11 days (range 7 - 14 days; SD=2). The products of conception were not karyotyped.

Pilot study of amnifiltration

All investigation of amnifiltration to date has been performed on pregnancies prior to elective termination and the procedure performed under general anaesthesia. Now a pilot study of amnifiltration in ongoing pregnancies is proposed.

Patients and methods

Twenty patients requesting prenatal diagnosis of fetal karyotype had

amnifiltration performed, using the same method as before (section 3.2.1). All patients were counselled about the procedure and gave their written consent; there was no randomisation. Prior to the procedure a detailed ultrasound scan was performed, fetal measurements collected and the gestational age confirmed. The study was approved by the hospital ethics committee. All patients were asked to report back if they experienced any vaginal bleeding or fluid leakage, and to re-attend for a follow up ultrasound scan at 20 weeks' gestation. The outcome of the pregnancies along with the neonatal details will be collected direct from the patient and/or her general practitioner.

Results

The indication for prenatal diagnosis was advanced maternal age in 18 cases and previous affected child in 2. The duration of pregnancy ranged from 76 to 92 days' gestation (mean=84.9 days). Amnifiltration was successfully carried out in all cases without difficulty with 24 mls amniotic fluid filtered. The mean harvest time was 12.6 days (range 9-15 days; SD=1.8). The karyotype illustrated a normal female in 11 cases and normal male in 8 cases, although one showed a pericentric inversion of the heterochromatin of one chromosome 9. One culture failed but a repeat amniocentesis was performed and demonstrated a normal female karyotype. All pregnancies have been rescanned at 20 weeks' gestation and 18 showed normal liquor volume, appropriate fetal size and no fetal structural abnormalities. In one patient there was detectable amnion membrane separation, with fluid still visible in the extra-amniotic space. She had not experienced fluid leakage and the fetus was appropriately grown and without structural abnormality. One patient noticed amniotic fluid leakage for the week post amniocentesis and

ultrasound examination illustrated decreased liquor volume one and two weeks after the procedure. However, by 18 weeks' gestation (6 weeks post procedure) the liquor and the fetus appeared normal. All pregnancies are ongoing.

3.3 Discussion

Filter type and pore size

The cells found in amniotic fluid measure between 2 and 60 μm in diameter (Bergstrom 1979, Gosden 1983), so it would be expected that a filter pore size smaller than 2 μm will trap all the cells. However, contrary to this expectation, the 1.2 μm pore size cellulose acetate filter allowed a significant number of cells to pass through (figure 3.2). One possible explanation for this is that the cell shape alters as the aspiration pressure draws it across the filter allowing it to squeeze through a relatively small pore. Under light microscopy viable amniotic fluid cells appear as large flat shapes with rolled edges and it is not difficult to imagine the cell rolling or folding up at the filter membrane surface and slipping through the small pores. Despite this seemingly traumatic passage the cells do not appear to be damaged as they can still initiate live cultures after filtration. An alternative explanation would be that the amniotic fluid leaked around the filter membrane because of poor fitting; however, if fluid leakage was the cause then it would affect all the filters not just the larger pore sized ones. As a result of these investigations the best filtration membrane to trap and allow retrieval of a maximum number of viable cells is the 0.8 μm pore size cellulose acetate filter.

The number of clones was chosen as the endpoint in these investigations because only undamaged viable cells capable of growth and thus suitable for karyotyping are of interest. The alternative of total cell number could be used to demonstrate ability to filter cells but would not reflect subsequent cytogenetic success.

Cell retrieval from filter surface and sterility

Once the cells have been trapped on the filter surface they can be successfully retrieved by either shaking or flushing them off the filter membrane. However, despite the relative success of agitating the filter, the resultant debris found in the culture tubes made examination of the clones difficult. Whereas cells retrieved by reverse flushing with culture medium produced no filter debris and 91% of the cells were retrieved with a single 10 ml flush. The flush method also allows the use of prepacked sterile filters; in contrast filter agitation demands a system where the filter membrane can be removed, making the equipment more difficult to assemble and increasing the possibility of infection. The system tested retains sterility, although this will be dependent on the care with which it is assembled.

Biochemical changes from filtration

These experiments demonstrated that despite using an organic filter membrane there was no influence on the concentration of urea, electrolytes and selected proteins in the amniotic fluid. Future studies should investigate the influence of amnifiltration on the concentration of larger protein molecules and bacteriostatic enzymes such as the lysozymes.

Circulating volume

Amnifiltration with recirculation of 20 mls of amniotic fluid can achieve a similar number of viable clones in culture as a 10 ml amniocentesis, but has the advantage that only one ml of amniotic fluid is removed. Indeed this volume can be reduced further if the fluid in the system dead space is re-injected after amnifiltration, in which case only the volume of fluid left in the needle would be removed. Fetal complications which result from fluid removal may thus be avoided, whilst taking full advantage of the culture success possible with early amniocentesis.

Temperature change

Where it was measured, patient's core temperature was consistently lower than normal body temperature, probably due to a combination of the anaesthetic agents (general anaesthetic for the termination of pregnancy) causing vasodilatation and the low room temperature. Despite this reduction, the amniotic fluid temperature remained higher than the core temperature, possibly due to increased heat production from the metabolically active fetus and placenta, or from slower heat loss. Perhaps an indication that amniotic fluid is important in removing heat from the fetus as has previously been suggested (Soothill et 1987). This finding is relevant to surgery in pregnancy, as prolonged procedures could result in substantial falls in amniotic fluid temperature. In such cases precautions such as warming blankets or higher ambient temperature should be used.

The effect of hyperthermia on the developing mammalian fetus has been studied extensively and it has been demonstrated that a rise of 2.5°C to 5.0°C for a minimum of one hour is required to induce congenital abnormalities (Edwards 1967, 1968, 1969, Edwards et al 1971). The pathophysiology of this is

not understood, but increased production of the so-called heat shock proteins may play an important role if the insult occurs at a critical period of organogenesis (German 1984).

The fall in amniotic fluid temperature with filtration was 0.6°C, a similar fall to the patient's core temperature. Indeed the temperature change could be reduced further by insulating or prewarming the apparatus, or by performing the procedure in a warmer ambient temperature. Whilst there is no conclusive data about the effect of reducing the fetal temperature, the reduction after filtration is relatively slight and may simply add to the physiological cooling function of amniotic fluid (Soothill et al 1987). Considering that this temperature drop may occur frequently in pregnancies where the mother undergoes a general anaesthetic, without obvious effect, one could predict that teratogenesis due to temperature change is unlikely.

Feasibility of amnifiltration

The results of the pilot study demonstrate the feasibility of amnifiltration for fetal karyotyping in ongoing pregnancies. Preliminary data suggest that the harvest time is similar to that with early amniocentesis (median 12 days) and in this respect it may not provide an advantage. Follow up at 20 weeks demonstrated viable pregnancies without evidence of fetal abnormalities. However, determination of the safety and diagnostic accuracy of the technique will necessitate a large prospective randomised trial comparing amnifiltration with the technique that proves to be the safest from the current ongoing trial of early amniocentesis versus CVS.

3.4 Amnifiltration: summary

1. Early amniocentesis removes a significantly high proportion of amniotic fluid (37% at 10 weeks), which in animal models has been associated with fetal pulmonary morbidity. Amnifiltration can achieve the same result but only removes 1 ml of amniotic fluid (3% at 10 weeks).

2. The most efficient filter tested was a 0.8 μm pore size cellulose acetate, filtering all the cells, whilst allowing their easy retrieval for culture.

3. The most practical method of cell retrieval from the filter surface is a reverse flush with culture medium. The majority (91%) of cells will be released by a 10 ml flush.

4. The amnifiltration system retained sterility in use, and no change in the biochemical components of the amniotic fluid was demonstrated as a result of filtration.

5. A minimum of 20 mls amniotic fluid must be filtered to establish a comparable result to a 10 ml early amniocentesis specimen.

6. The core temperature of anaesthetised patients was low. Amnifiltration performed in a low ambient temperature caused a fall in the amniotic fluid temperature of the same magnitude as the drop in anaesthetised patient's core temperature. The procedure should be carried out in a warm environment, to limit this change.

7. Filtered cells can be successfully cultured and karyotyped, in a similar time interval to that of early amniocentesis. There is no evidence that filtering the cells shortens the culture harvest time, as initially predicted.

Chapter 4

Conclusions and recommendations

Chapter 4 Conclusions and recommendations

4.1 Conclusions

Chromosomal abnormalities are common and are associated with a high incidence of fetal and postnatal death, as well as major handicap in survivors. The diagnosis of chromosomal abnormalities constitutes the main indication for invasive prenatal diagnostic techniques. Although amniocentesis at 16 weeks gestation is now well established with relatively low risk of spontaneous abortion (1%), neonatal respiratory morbidity (1%), false positive (0.06%-0.4% cases) and false negative (0.06%-0.6% cases) results, early reports had suggested the risks were much greater. The introduction of first trimester diagnosis by CVS was advantageous but currently there is controversy as to the safety and diagnostic accuracy of this technique. The extent to which, in historic terms, recent concerns about CVS will prove to be unjustified is uncertain. It is therefore important that the technique continues to be evaluated but also that new techniques are introduced.

The data of this thesis have provided evidence that amniocentesis can be performed in the first trimester of pregnancy. Furthermore, the ability to obtain a sample and achieve a cytogenetic result at 10-14 weeks' gestation is similar to that for CVS. Although the total number of cells in amniotic fluid increases with advancing gestation, the number of live cells at 10-14 weeks is similar to that at 15-18 weeks. Before 10 weeks and/or when the fetal crown-rump length is less than 38 mm the number of live cells and the chances of successful culture are dramatically reduced. This thesis has therefore defined

the entry criteria for a major prospective randomised study to compare the safety and cytogenetic accuracy of first trimester amniocentesis with CVS. Preliminary results from the outcome of the first 650 cases suggest that the rate of livebirths following early amniocentesis and CVS is similar (92% versus 93.8%), but that the fetal loss rate is higher after early amniocentesis (5.2% versus 1.2%).

One of the concerns with early amniocentesis is that it removes a high proportion of amniotic fluid (37% at 10 weeks), which in animal models has been associated with fetal pulmonary complications. This thesis, through a series of experiments, has established the feasibility of amnifiltration whereby amniotic fluid cells are obtained for culture and cytogenetic analysis with minimal loss of amniotic fluid volume. The best filter found to perform this, is constructed of cellulose acetate with a 0.8 μm pore size; cells can easily be retrieved by a reverse flush with culture medium. A minimum of 20 mls of amniotic fluid must be filtered to obtain the same cytogenetic result as a 10 ml amniocentesis specimen. The system retains sterility and does not alter the basic biochemical composition of the amniotic fluid. The system does, however result in a temperature drop in the amniotic fluid which may be decreased by performing it in a warmer environment.

4.2 Criticisms

This thesis has investigated and established the feasibility of amniocentesis and amnifiltration at 10-14 weeks' gestation as alternative methods to CVS for first trimester fetal karyotyping. However, the application of these

techniques will require examination of short-term and long-term complications as well as diagnostic accuracy. On the basis of currently published reports the overall risk of fetal loss following first trimester CVS is approximately 4% (Canadian collaborative trial 1989). On the assumption that the fetal loss rate following early amniocentesis would be 1% less, 6,700 patients would need to be recruited into the study to prove that early amniocentesis is significantly safer than CVS ($p<0.05$ with 80% confidence). Consequently, the outcome results presented in this thesis must be considered preliminary.

4.3 Future studies

1. Randomised trial of early amniocentesis and chorion villus sampling to determine the safety and diagnostic accuracy of the two procedures.

This thesis has proven the feasibility of early amniocentesis, it is now imperative to establish the safety and diagnostic accuracy of this new technique. To do this it could be compared to no procedure at all, but parents requesting prenatal diagnosis are unlikely to join a study which includes the possibility of no test. So the most realistic design is that discussed in section 2.3. This is a prospectively randomised comparison of early amniocentesis and CVS, performed over the same gestational range (10-14 weeks), using the same methods and carried out by operators equally skilled in both techniques. The study is thus a comparison of the risks of inserting a needle into the placenta whilst avoiding the amniotic sac, with the risks of inserting a needle in the amniotic sac whilst avoiding the placenta.

The population to be studied must be carefully considered, as although the indication for the procedure will be prenatal diagnosis of fetal karyotype, there are wide variations in the risk of parents having an affected pregnancy. The risk can be low, as in indications like advanced maternal age, or high as in parental balanced translocation (Table 1.3). Since eventually the techniques will be compared for total fetal loss rate (MRC 1991), which will include therapeutic terminations, if one group has a higher proportion of patients with a high genetic risk this will bias the number of terminations and therefore, fetal losses between the groups.

In addition to fetal loss rate the trial should address the question of diagnostic accuracy and postnatal morbidity. Confined placental abnormality is the major cause of false diagnoses in CVS samples, as the placental karyotype may differ from the fetus. This is less likely in conventional amniocentesis where the cells are derived from fetal tissues. However, this may not be the case in early pregnancy where a proportion of cells could be derived from amnion or trophoblast (Nielson and Gosden 1991). All pregnancies undergoing termination, should have confirmation of the fetal karyotype by cytogenetic analysis of fetal tissues. All infants should be examined by a paediatrician for the detection of any defects and lung function tests should be undertaken, in a sample, to investigate the possible effect of the prenatal diagnostic techniques on pulmonary development.

2. Randomised trial of amnifiltration versus either early amniocentesis or chorion villus sampling.

To demonstrate if the smaller volume of amniotic fluid removed at amnifiltration is associated with a lower risk of both short-term and long-term complications amnifiltration should be compared to either amniocentesis or CVS (whichever proves to be safer in the first study) in a prospective trial as described above.

3. Amnifiltration at less than 10 weeks' gestation.

In this thesis it was aimed to establish the minimum volume necessary to achieve a cytogenetic result at 10-14 weeks. Future studies could investigate the feasibility of fetal karyotyping by amnifiltration at less than 10 weeks.

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