REVIEW ARTICLE

Deciding by numbers: the genesis of prenatal serum screening cut-off limits for Down syndrome and neural tube defects

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ABSTRACT

The use of maternal blood markers and early ultrasound measurements to screen for fetal neural tube defects and fetal aneuploidy (primarily trisomy 21, Down syndrome) is now a well-established practice in many countries. However, the origins and background relating to how the techniques became established and how the various action limits were derived is not universally known or understood. In this paper, we consider the historical origins of the maternal blood screening tests, the establishment of the analytical parameters currently in use, the development of the nuchal translucency test and the integration of multiple analytical parameters to facilitate the diagnosis of fetal abnormalities.

Key words: prenatal diagnosis, Down syndrome, neural tube defects, chromosome aneuploidy, cut-off limits.

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INTRODUCTION

Historically the outcome of a pregnancy was unknown, with the majority of parents anticipating a trouble-free pregnancy and birthing resulting in a healthy child. For some parents, however this was not the outcome and the child was born with a disability ranging from mild to severe. While certain disabilities could be corrected, or treated there remained a group of disorders for which there was little or no correction such as chromosome disorders, severe neural tube defects and certain inborn errors of metabolism.

It is interesting to note that the understanding of human chromosomes, neural tube defects and inherited metabolic diseases has evolved over the last 50 years. For example, in the mid-1950s there was considerable debate regarding the number of human chromosomes ranging from 46,47 and 48 (1,2,3)' with a final (and correct) resolution of 46. This resolution led to the discovery that Down syndrome was the result of inheriting an extra chromosome 21 giving rise to the terminology "trisomy 21" (4). Notwithstanding this discovery, there is historical evidence of Down syndrome, which pre-dates the publication of John Down in 1866. Both visual (as early as 1500BC) and archeological evidence (ca 5-6th century) respectively indicates that children with Down syndrome have a long historical lineage (5,6). Equally neural tube defects can be dated back to at least ancient Egypt with some archeological evidence from 10,000BC (7).

Despite this long history, Down syndrome and neural tube defects remained "an accident of birth" until the advent of second trimester amniocentesis. Limited amniocentesis had been used to detect and monitor haemolytic disease of the newborn (Rhesus disease) in the third trimester of pregnancy (8.9). Later, it was demonstrated that fetal chromosome analysis could be undertaken using amniotic (shed fetal) cells (10). These diagnostic landmarks brought about significant changes in perceptions, attitudes and diagnosis of Down syndrome and other fetal chromosome abnormalities, which was reinforced by the introduction of obstetric ultrasound in the 1970s. This offered for the first time, the opportunity to test for and identify fetal chromosome disorders at 14 to 16 weeks of pregnancy thereby fitting the time frame in the UK for termination of pregnancy. As there is a strong association of fetal Down syndrome and other chromosomal disorders occurring in women over the age of 35 years (discussed in more detail later) amniocentesis provided a powerful diagnostic tool in early pregnancy. This was subsequently extended to diagnose certain inherited metabolic disorders (11). However, as women are deferring pregnancies until later i.e. 35 years or older, a progressive increase in the incidence of Down syndrome is now being observed (12).

Although amniocentesis provided a reliable diagnostic procedure, it is invasive and has associated risks especially in the early days prior to the use of ultrasound and was time consuming. A major change in approach to prenatal diagnosis occurred in 1973 when a-fetoprotein was discovered in maternal blood and elevation of the protein had a strong association with fetal neural tube defects (anaencephaly and severe spina bifida) in early pregnancy (13). Subsequently low maternal serum a-fetoprotein levels in maternal blood were identified as having a strong association with fetal Down syndrome (14,15) thereby providing a screening test for Down syndrome for all women from an ante-natal blood sample in early pregnancy. Further research identified other maternal blood biochemical markers, which increased the accuracy of the screening test and the subsequent development of unified cut-off or action limits to identify pregnancies associated with Down syndrome. In this paper, we describe the development of the ante-natal screening programme and discuss the evolution of the action or decision making limits used in the maternal pre-natal screening programme.

BACKGROUND

At conception and early embryonic development humans have a high rate of embryonic wastage primarily due to chromosome abnormalities with an estimated loss of approximately 70% of all embryos within six weeks of fertilization (16,17). As the pregnancy proceeds some chromosomal abnormalities persist and some will result in early fetal loss as a miscarriage (18). Amongst the most common of the chromosome abnormalities that continue during pregnancy are trisomy 21 (Down syndrome), trisomy 18 (Edward syndrome); trisomy 13, (Patau syndrome); Turner syndrome (typically inheritance of one X chromosome instead of two) and triploidy (more than one set of

chromosomes). Normally humans have 46 chromosomes (23 from each parent including either two X chromosomes (female) or XY chromosomes (male). These are all balanced with approximately equal contribution from each parent. In trisomy, an additional chromosome has been inherited such as three chromosomes 21 in the majority of cases of Down syndrome and creates a case of where more is not beneficial. In general, the term used to indicate that there are errors in chromosome numbers is aneuploidy but does not indicate a specific number or condition. One of the significant risk factors for fetal chromosome abnormalities is maternal age, with increasing maternal age strongly associated with increased risk of fetal chromosome abnormalities (19). Typically, at the age of 20 there is a risk of any fetal chromosomal abnormality of 1 in 526, by 35 this has changed to 1 in 192, and by 40 it is 1 in 66 (20). However, the most common fetal chromosome abnormality at birth is Down syndrome with an overall incidence of 1 in 800 live births (21) But the risk varies considerably with age, for example at age 20 the risk is 1 in 2300, however over the age of 45 the risk is 1 in 40 (21). This increasing risk of fetal chromosome abnormalities associated with maternal age has given rise to the development of techniques to identify potentially 'at risk' reproductively older women (over the age of 35 years) and provide diagnostic services to identify or reassure fetal normality. In addition to fetal aneuploidy, fetal neural tube defects (primarily anencephaly and spina bifida) at an incidence of approximately 1 in 1300 births are potential causes of fetal abnormality but there is some population variation (22) and prenatal detection could provide a useful diagnostic technique in identifying pregnancies affected by these abnormalities.

PRENATAL TESTING – BACKGROUND

Prenatal genetic testing has made significant progress since its early introduction in the 1970s when fetal chromosome analysis became feasible with the development of amniocentesis and the ability to culture fetal cells present in the amniotic fluid sample. This service was available from 1975 in Christchurch and provided an analytical service for both the North and South Island (23). Prior to the advent of ultrasound, the amniocentesis procedure was not without risk and the risk of penetrating the placenta or the fetus with the needle was always a possibility. Miscarriage risk was always a complication; however, with the advent of obstetric ultrasound both placenta and fetus could be localized with the associate reduction in risks to the fetus. Ultrasound also reduced the risk of maternal blood contamination of the amniotic fluid sample thereby increasing the accuracy of the fetal chromosome analysis by eliminating the possibility that the chromosomes were from the mother. The development of prenatal chromosome analysis was viewed as providing a 'choice' especially for those women over the age of 35 years where the risk of a fetal chromosome abnormality starts to increase significantly (21). A culture within the medical and scientific community evolved relating to 'prevention' and many women were offered prenatal chromosome analysis from which the outcome was termination of the fetus that had a karyotype associated with known pathology. This was significantly reinforced with discovery of a-fetoprotein (AFP) in amniotic fluid in 1972 and subsequently in the maternal blood in 1973 (13,24) with the significant association of elevated AFP with neural tube defects (anencephaly and spina bifida). This discovery was a milestone in the advancement of prenatal diagnosis at a time when obstetric ultrasound was still in its infancy. For the first time a biochemical analysis could reliably predict fetal outcome without the need of fetal cell culture and maternal serum screening using AFP became part of the obstetric management, often used in conjunction with amniocentesis. A major advantage of AFP testing was that the optimum time for diagnostic use (15 to 19 weeks-gestation) coincided with the optimum time for fetal karyotype analysis and fitted comfortably with the legal time frame for termination of pregnancy in the United Kingdom, where the research was conducted. However, there was variability in interpreting individual results within the reference ranges for AFP both in amniotic fluid and maternal serum largely due to variation in methods used in different testing centres, which was overcome by a statistical manipulation of reference ranges, rather than use the conventional statistical technique of percentiles or standard deviation around the mean. Subsequently it was proposed that results could be expressed in relation to multiples of the median (MoM) linked with gestational age (25). Intensive research ensued to seek new biochemical markers of fetal disorders and to improve the reliability of the AFP testing. Whilst there was a clear association with elevated AFP and the neural tube defects data emerged that there was another cohort of AFP results that were in the lower region of the maternal serum AFP values and were identified to be associated with fetal chromosome abnormalities (14,15). Analysis and refinement of these data identified a correlation between low maternal serum AFP and Down syndrome (14). Subsequent research provided a risk estimate for Down syndrome using low AFP values and maternal age (26).

DIAGNOSIS BY NUMBERS

The advent of numerical data to undertake prenatal diagnosis was a major shift in concepts of diagnostic techniques in obstetrics. Karyotype analysis was visual, early (first and second trimester) examination of the fetus essentially relied on the 'laying on of hands', reliable gestation dates became essential for the accurate interpretation of the biochemical results, and diagnosis shifted from clinician based to laboratory based information systems. Paralleling the development of the biochemical diagnostics was the development and implementation of the use of ultrasound to measure fetal biparietal diameters and crown rump length, which correlated with gestational age thereby providing accurate gestational dates to assess the biochemical results. Although an accurate independent technique could be used to establish gestational age, another issue arose that different laboratories had variable reference ranges for AFP results, raising the possibility of misinterpreting diagnostic results. This dilemma was largely overcome by using the multiple of the median. Using this technique, the individual result is assessed as to how far it deviates from the median, this normalized the result and provided a technique to compare results both from individual diagnostic centres but to also undertake retrospective analysis of data. Threshold levels could be established as multiples of the median for a given gestational age, where the most likely cut off would detect the maximum number of neural tube defects (25). Re-analysis of data from the prenatal diagnosis of NTD revealed a set of data with very low maternal serum AFP levels (contrary to the high levels associated with NTD) these data were found to correlate with fetal chromosome abnormalities (15,27) and the possibility of a maternal serum marker as a screen for Down syndrome quickly became a reality, with the finding initiating a new analysis of numerical data (26). As the AFP data was now in the form of MoM large multi-centre collaborative studies were undertaken with pooled data confirming the original reports. As the incidence of Down syndrome is linked to advancing maternal age a regression curve model was developed, which integrated the risk factor for increasing frequency of Down syndrome with the maternal serum AFP value (26). Additional discovery of maternal serum biochemical markers associated with Down syndrome and other fetal chromosome abnormalities continued with elevation human chorionic gonadotrophin (βhCG) values associated with Down syndrome (28), low maternal blood unconjugated oestriol (29) and the development of the 'triple screening test' for Down syndrome integrating maternal serum AFP, βhCG and unconjugated oestriol (30). Using the triple screen plus ultrasound dating and the risk factor associated with maternal age >39 years the expected detection rate for Down syndrome was predicted to be 93% with a false positive rate of 24%, which increased with advancing maternal age (31). In 1992, a fourth marker maternal blood marker was identified to correlate with the antenatal prediction of a Down syndrome fetus, Inhibin A, which is a placental protein and is elevated above the median in the majority of cases of Down syndrome (32).

This led to the development of a four-marker biochemical screening system utilizing ultrasound, AFP unconjugated oestriol, βhCG and Inhibin A. Taken overall the detection rate was 92% and the false positive rate improved to 19.1% (33). A further placental protein was identified as a potential maternal serum marker. Pregnancy-associated plasma protein A (PAPP-A), which was decreased in both Down syndrome and other chromosomal abnormalities in the first trimester. (34). Decisions had to be made on the most appropriate time to screen, which markers had the greatest utility and statistical modeling to define cut-off limits appropriate for the stage of gestation. Using a meta-analysis approach, it was determined that screening in weeks 9 to 11 using maternal serum PAPP-A, free βhCG, and unconjugated oestriol a detection rate of 65% was predicted with a false positive rate of 5% and if ultrasound nuchal translucency (see next section) was included the combined prediction rate rose to 88.3% (35). Various mathematical models were applied to the maternal serum markers relating to the best possible diagnostic combination and the combination across the first and second trimester of nuchal translucency, PAPP-A, AFP, unconjugated oestriol, βhCG and inhibin-A gave a 93% detection rate with a false positive rate of 2.6% (36). The overall screening results have been analysed in the UK in two programmes; SURUSS (Serum, Urine and Ultrasound Study) and FASTER (First and Second Trimester Evaluation of Risk Trial), which indicated either the first or second trimester integrated screening progrmames were effective but did not support the use of individual screening tests (37).

NUCHAL TRANSLUCENCY

This is classed as a 'soft' marker for fetal aneuploidy as it is non-specific and often transient. The nuchal translucency can be visualized using ultrasound in both normal and aneuploidy fetuses, and must be used before 14 weeks gestation as it can no longer be seen by the end of the second trimester and nuchal fold measurement would be required (38,39). In Down syndrome and other aneuploidies the nuchal translucency behind the neck of the fetus 'thickens' due to an accumulation of fluid and about 20% of fetuses with increased nuchal translucency will have chromosomal abnormalities (40) with the incidence increasing as the nuchal translucency thickens. However, this is not a specific test and increased nuchal translucency has been reported in congenital heart defects, certain autosomal disorders and some structural abnormalities (41,42). There is some debate relating the most appropriate cut-off in relation to the 95th or 99th centile, however, a consensus is that above the 99th centile (3.5mm) represents an increased nuchal translucency (38,43). Used in conjunction with the biochemical markers across first and second trimester and factoring maternal age, the combination of nuchal translucency, PAPP-A, maternal serum AFP, unconjugated oestriol, βhCG and inhibin-A gave a 93% detection rate with a false positive rate of 2.6% (37).

WHAT CAN GO WRONG?

As with all biochemical tests there are factors, which will influence the result and the use of the above maternal blood screening tests are no exception. The mathematical calculation of risk is complex involving maternal age, multiple analytical parameters, the conversion of data to MoMs and a comparative analysis to a population based model using multivariate Gaussian statistics. This relies on the use of software of which there were a number of versions some of which did not give consistent comparative results (44). In addition, although MoMs have been generally accepted as a normalizing method, questions arose about the universal acceptance of MoMs due to how differing centres calculated their threshold values and considered that many threshold MoM values related to differing percentiles of gestational age–dependent distributions (45). Additional factors were identified as introducing variance of the results. Variable results from AFP, βhCG and unconjugated oestriol from four population groups in the USA, (Caucasian, Afro- Caribbean, Hispanic and Asian) were identified and although this variability did not bias screening for Down syndrome it was considered that higher normal values for serum AFP in Asian populations may have to use adjusted median values (30,46). An additional report indicated that smokers may also have to use separate MoMs as they identified that smoking modified the risk estimate for Down syndrome from 1 in 250 to 1 in 200 when compared with non-smokers (47). Later research indicated that smoking reduced the serum PAPP-A by 15% in the first trimester and βhCG by 18% in the second trimester which may impact on the mathematical modeling of MoMs (46). Serum AFP, βhCG and unconjugated oestriol are lower in Type 1 diabetes which may require adjustment to the MoMs to provide a reliable risk prediction (48). Result adjustment for maternal weight as a compounding factor for serum AFP has been proposed but not a significant influence on the AFP (49). At the time, no reliable data was available for multiple pregnancies and adjustment of data was considered unreliable in predicting fetal risk and first trimester nuchal translucency was considered to provide the only test for these pregnancies. Notwithstanding the issues outlined above, other factors relating to fetal distress, death, miscarriage, etc. will all influence the biochemical marker results and possibly skew the interpretation of risk. Although nuchal translucency and nuchal fold measurements rely on biophysical data concern has been expressed that the training and quality of results may lead to unsatisfactory measurements and potential misinterpretation especially in situation where nuchal measurements are being used in isolation (50,51).

WHY SCREEN FOR NEURAL TUBE DEFECTS AND FETAL ANEUPLOIDY?

What constitutes a NTD? These are defects, which occur during early embryo development when the area (the neural tube) in the embryo is destined to become the spine, spinal cord, brain and skull are formed. Failure to close at the specified time during embryogenesis can result in a wide range of abnormalities ranging from anencephaly (failure of the top of the skull and brain to develop) to varying degrees of the spine failing to close (spina bifida) of which meningomyelocele (failure of the posterior or caudal part of the spine to close) is the most common (52). In a Caucasian population, the incidence of neural tube defects is around 1 in 1300 live births, however this estimate varies with ethnic origin and geographical location, for example: in Northern China the incidence is 9 per 1000 and in Japan 0.6 per 1000 births. Analysis of birth records in Christchurch, New Zealand between 1970 to 1975 indicated that the overall incidence of neural tube defects was 5.86/1000 births (53). The majority of NTDs are non-syndromic and although genetic factors are considered to influence NTDs there is also an environmental consideration and a link with maternal age (22,54). This is indicated in Ireland where the incidence of NTDs in 1974 was 8 per 1000, however in 1994 the incidence was 3.3 per 1000, which has been associated with a significant increase in folate (a water-soluble vitamin strongly associated with decreasing incidence of NTD) the in the diet (52,55). Folate therapy however, must be commenced prior to becoming pregnant and cannot correct an existing fetal neural tube defect. The ability to screen in early pregnancy to detect the more severe forms of NTDs provides the intending parents options on deciding pregnancy outcomes and possible future care of a child with a disability.

Aneuploidy is an error in cell division whereby the resulting daughter cells contain the incorrect chromosome number, either extra or less. In the majority of cases the fetus will not survive in-utero and will miscarry (56). Those that do survive to birth will have defects associated with the incorrect chromosome number, which accounts for 0.16 to 0.27% of live births in humans (56). The most well-known of which is Down syndrome. Typically, infants with Down syndrome may have heart defects, thyroid gland dysfunction, digestive tract problems, facial and hearing defects as well as intellectual disability and repeated infections. Unfortunately, the prenatal diagnosis of Down syndrome cannot indicate the severity of the intellectual

disability associated with trisomy 21; however, heart and other structura l defects are often identified during prenatal ultrasound scans. Trisomy 18 (Edward syndrome) which affects 1 in 3000 live births, however, is associated with a high rate of fetal and neonatal death as well as multiple abnormalities and those that do survive have severe to profound developmental disabilities (57)). Trisomy 13 (Patau syndrome), which affects 1 in 5000 infants, may survive beyond birth to one year but will die due to the multiple abnormalities associated with this disorder. As pregnancies are deferred and maternal age increases so will the associated risk of fetal aneuploidy and the necessity to consider options at a late stage in a woman's reproductive life.

THE FUTURE OF PRENATAL DIAGNOSTICS

Although severe NTD can now be identified using ultrasound and the techniques are becoming increasingly sophisticated and sensitive in identifying fetal abnormalities, it still does not provide a definitive fetal diagnosis for chromosome abnormalities. Currently the two most reliable techniques are chorionic villus sampling and amniocentesis with fetal karyotype analysis. These are close to 100% accurate but clearly carry risk, particularly of miscarriage. New technologies however, could well eliminate these risks and provide safer options in prenatal diagnosis. It was established over 20 years ago that fetal cells were detectable in the maternal circulation often as early as six weeks' gestation (58) and that fetal DNA can be detected in the maternal circulation giving rise to speculation that this discovery could lead to the development of non-invasive prenatal diagnosis (59). Subsequent work using ivf and embryo replacement, established that fetal DNA could be detected in the maternal circulation as early as day 18 post-conception and concluded that the fetal DNA detected resulted from the trophoblast as the fetal circulation proper is not established until around day 28 post conception (60). However, other workers have indicated that the fetal DNA exists in fragments 80% of which are less than 200bp and represents approximately 6 per cent of the circulating cell free DNA in the maternal circulation, the remainder being maternal (61). Notwithstanding the disproportionate relationship between fetal and maternal cell free DNA reports from large population cohorts indicate that use of fetal cell free DNA for identifying the most frequent fetal trisomies (13,18 and 21) had a higher predictive value than serum biochemical and nuchal transparency analysis (62,63). Further refinement of the fetal cell free DNA techniques has indicated that identification of fetal microdeletion syndromes was possible (64). These microdeletions are independent of maternal age and would not normally be detected using current cell free fetal DNA (cfDNA) analysis or the normal techniques used in chromosome karyotyping (64,65).

The potential of non-invasive prenatal testing (NIPT) for fetal aneuploidy using cfDNA is already raising questions however relating to patient information, technical competency, limitations on identifying relevant clinical conditions, and ethical concerns (66). In addition, it has been proposed that in maternal low-risk populations the positive predictive value was found to be less than 50% (62). The Society of Maternal-Fetal Medicine (2016) has recommended that the cell free DNA technology should not be used for low-risk pregnancies and that cfDNA screening results should be confirmed by an alternative technique such as chorionic villus sampling (67). Further research has identified that here is a possibility of the detection of occult maternal malignancies that may be discordant with the fetal karyotype and these results required follow-up (68). An alternative consideration is the presence of circulating fetal cells in the maternal blood that are detectable from approximately seven weeks of pregnancy and can persist for years (69,70). The ability to isolate such cells provides an alternative opportunity to isolate fetal DNA and analyze it using molecular biology technologies and bioinformatics techniques. A more exciting future is the ability to 'switch off' the expression of the extra chromosome in the trisomies. Recent research using cell culture has demonstrated that it is possible (*in-vitro* at least) to silence the additional chromosome 21 associated with Down syndrome using a mechanism that normally silences one of the X-chromosomes in women (71,72). Whether this may translate in to a future treatment and the implications associated with human genome modification still remain to be considered as has been recently highlighted with the molecular 'engineering' of non-viable human embryos (73,74).

DISCUSSION

Scientific developments in prenatal diagnosis have made a major contribution to the identification of fetal abnormalities and genetic diseases at a time when decisions can be made on pregnancy outcomes. For many this has meant the termination of the pregnancy when an affected fetus has been identified. However, central to the use of early pregnancy prenatal screening is the issues relating to understanding the nature of the procedures, consent and the decision-making processes. Over time the concept of prenatal diagnosis has changed from 'prevention' to offering choices of pregnancy outcomes. What is the nature of 'consent' when ultrasound scans and 'routine' blood samples are taken and testing initiated? This concept is critical in the use and acceptance of the screening procedures. For example, if the results of the screening indicate an abnormality such as a fetal aneuploidy, will the woman proceed to chorionic villus sampling or amniocentesis to verify or otherwise the screening result? In a reproductively older woman with a first pregnancy will the risks of a miscarriage (albeit small) out-weigh the potential benefit? Entering in to a prenatal screening programme the outcome must be understood in order that autonomous choice and informed consent are within an ethical and legal framework. A survey of New Zealand maternity carers identified that there was strong support for the introduction of NIPT in the first trimester; however, of particular concern was that of the 108 maternity carers interviewed, only 20% would refer to a genetic counselor, and the majority would provide support themselves, raising questions relating to whether there is relevant expertise within that group (75). In addition, it is important to consider individual beliefs and cultural norms in relation to the information provided and the management of the results and subsequent out comes resulting from prenatal testing (76). The decision-making process based on a set of complex data analysis will for many be difficult to reconcile especially when the expectation is a routine clinic visit which may not have any other personal support and time is not on the side of extensive consultation.

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