Incidence of sex chromosome aneuploidy in Northern Italy: 27-year longitudinal study

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CONTRIBUTION

What are the novel findings of this work?
This longitudinal, population-based study demonstrates that the rate of diagnostic invasive prenatal testing has decreased, while the prenatal diagnosis of SCAs has increased. Based upon reported indications for diagnostic prenatal testing, the increase in prenatal ascertainment of SCAs has been influenced by the availability of cfDNA.

What are the clinical implications of this work?
Without a sensitive prenatal screening tool, ascertainment of individuals with SCAs, particularly in infancy, is challenging due to the paucity of physical findings and non-specific behavioral symptoms. Including SCAs in prenatal cfDNA screenings will result in the earlier identification of affected individuals and improved outcomes.
ABSTRACT

Objectives: The availability of cell-free DNA (cfDNA) as a screening tool affords an opportunity for non-invasive identification of sex chromosome aneuploidies (SCAs). This longitudinal study from 1995 through 2021 investigates both the evolution and frequency of prenatal diagnostic testing using amniocentesis (AF) and chorionic villus sampling (CV), and the detection of SCAs through cfDNA samples from a large cohort in Northern Italy.

Methods: The results of genetic testing from CV and AF samples collected from public and private centers in Italy from 1995 to 2021 were collected. Chromosomal analysis was performed by routine Q-banding karyotype. Regression analyses and descriptive statistics were used to determine population data trends regarding the frequency of prenatal diagnostic testing and the identification of SCAs and correlated with changes in the indications for prenatal diagnostic tests and available screening options.

Results: In 27 years, 13,939,526 births and 231,227 invasive procedures were performed. This resulted in the prenatal diagnosis of 934 SCAs. After the commercial introduction of cfDNA use in 2015, the frequency of invasive procedures significantly decreased ($P=0.03$), while the frequency of prenatal SCA detection significantly increased ($P=0.007$). The indication for invasive procedures also shifted from advanced maternal age (AMA) to positive cfDNA results for sex chromosome trisomies (SCTs).

Conclusions: Our findings suggest the inclusion of SCAs in prenatal cfDNA screening tests can increase the prenatal identification of affected individuals. As the benefits of early ascertainment are increasingly recognized, it is essential that healthcare providers are equipped with comprehensive and evidence-based information regarding the associated phenotypic differences.
and the availability of targeted effective interventions to improve neurodevelopmental and health outcomes for affected individuals.
INTRODUCTION

Since the 1960s, prenatal diagnosis of chromosome abnormalities has been possible using amniocentesis (AF) for common trisomies and for older mothers in the second trimester. Chorionic villous sampling (CV) was introduced in the 1980’s with the advantage of being performed in the first trimester. Screening tests for T21, T18 and T13 include a first trimester option combining ultrasound measurement of the nuchal translucency (NT) with serum markers, often referred to as the First-trimester Combined Test (FCT). With the reduction in AF and CV procedures, a decrease in the number of sex chromosome aneuploidies (SCAs) identified prenatally was observed.

Since 2011, the use of cell-free DNA (cfDNA) of placental origin in the maternal plasma has been increasing as a primary screening modality, with the option to include the sex chromosomes, expanding the scope of conditions amenable to non-invasive prenatal screening. Currently available cfDNA tests, also called noninvasive prenatal screening/testing (NIPS/T), evaluate the presence of autosomal trisomy for chromosomes 13, 18, and 21 with detection rates of 99.0%, 97.9%, and 99.7%, respectively, and a false positive rate (FPR) of 0.04%. Sensitivity and FPR for 45, X were 95.8% and 0.14%, respectively. The SCTs were combined with a sensitivity of 100% and FPR of 0.004%. Metanalysis study reported sensitivity/specificity for 45, X of 93.9%/99.6%, 47, XXX 76.2%/99.5%, 47, XXY 82.9%/99.9% and 47, XYY 91.7%/100%. Positive predictive values (PPV) for SCAs large Italian survey are 27%, 62%, 72% and 96% for 45, X, 47, XXX, 47, XXY and 47, XYY respectively. The overall PPV for 45, X is significantly lower than for SCT. This 27-year longitudinal population study describes the impact of cfDNA screening upon the frequency of prenatal diagnostic testing and detection of SCAs in a large, unselected cohort.
METHODS

Inclusion criteria

This is a longitudinal retrospective study of prenatal diagnostic testing samples received and analyzed by TOMA Advanced Biomedical Assays S.p.A., a single genetic center, (Impact Lab), between 1995 and 2021. TOMA laboratory received samples from a variety of public and private prenatal diagnostic centers located mainly in Northern Italy. Cytogenetic services provided to public and private centers affiliated with the national/regional health system were reimbursed through government/regional health plans.

Each sample was accompanied by a requisition form, including a concise, complete description of the fetal phenotypic features from the ultrasound and the indication for prenatal diagnosis that was ascertained by the physician who performed the invasive procedure. The study was approved by the TOMA Institutional Review Board (2020/27). Birth rates in Italy were obtained from the Italian National Institute of Statistics (ISTAT).13 AF and CV samples identified with a mosaic aneuploidy or other rare SCAs (e.g.: 48, XXXY, 49, XYYYY and other variants) were excluded. Multiple gestations were also excluded and only singletons were evaluated.

Karyotype and cytogenetic analysis

Karyotype analyses of AF and CV samples were performed using procedure and evaluation criteria following Italian guidelines.14 These were progressively updated during the study period, in accordance with European guidelines.15,16

Standard protocols were used to set up the cultures and chromosome preparations, and Q-banding technique (QFQ) was used for the entire cohort. Karyotype results were formulated

The methods of cytogenetic analysis have been described in detail in a previous publication.18 Briefly, CV sampling was performed by combining cytogenetic analysis via direct preparation (Dir; cytotrophoblast) with long-term culture (LTC, mesenchyme). A total of at least 16 metaphases were analyzed. Karyotyping of AF samples was performed by the in-situ method (on at least 10 metaphases, derived from 10 colonies from at least two independent cultures). A homogeneously aneuploid cytogenetic result was diagnosed when the same aneuploidy was observed in all analyzed metaphases.

**Time periods**

Changes in clinical practice in Italy occurred during the study, primarily based upon changes in professional care guidelines and the commercial availability of different aneuploidy screening methods. To determine the effect of these changes, three time periods were defined. Between 1995 and 2003 (period 1), CV or AF assessment was primarily offered to women of AMA. Between 2004-2015 NT and FCT were broadly available and offered (period 2). Finally, cfDNA was consistently available to women of all ages beginning in 2016, defining period 3 (2016-2021).

**Indications for diagnostic testing**

The indications for diagnostic testing were abstracted from the requesting provider, with the following categories; advanced maternal age (≥35y; AMA); anxiety (<35y); cfDNA (high risk or inconclusive); increased nuchal translucency (NT), cystic hygroma (CH) and hydrops;
ultrasound scan abnormality excluding NT, CH, hydrops (US anomaly); increased or intermediate risk with another traditional screening method combining NT and serum analytes; intrauterine fetal death (IUFD); Other (e.g.: positive family history, parent carrier of a balanced chromosome abnormality, risk for monogenic disorder, IVF pregnancy, infectious disease, indication not specified). If an SCA was identified by AF performed after a mosaic CV result, the indication for the CV was used.

**Statistical analysis**

During the period of 1995-2021, the frequency with which CV/AF identified an SCA was determined on an annual basis. The number of CV/AF samples were evaluated as a function of the number of live births and compared for trends using the Statistical Package for Social Sciences (SPSS). A regression analysis was performed to evaluate the trend of the data year by year and for each of the three time periods. We then determined the number of cases of 45, X, 47, XXX, 47, XXY and 47, XYY identified by CV/AF each year. The data was evaluated for all SCAs combined, 45, X separately, and the SCTs combined. The indications for prenatal diagnostic testing were compared for the three time periods using SPSS statistical software. MATLAB R2019b was used to generate the figures. Spearman and Pearson correlation tests were performed to investigate the correlation between the changes in the approach to prenatal screening, diagnosis in the three time periods, and the detection of SCAs. *P*-values < 0.05 were considered significant.
RESULTS

Between 1995-2021, there were 13,939,526 live births and 231,227 diagnostic tests reported by the laboratory. Significant changes in the live birth rate in Italy were noted. The highest number of live births, 576,659, was observed in 2008. This was followed by a steady decline in the annual birth rate, with a low of 395,000 in 2021. Similarly, the number of diagnostic test samples received by the laboratory declined. Figure 1 illustrates the number of tests (CV and AF) performed per number of live births over the years of 1995 to 2021. Figures 2 and 3 illustrate the number of CV and AF tests performed per number of live births over the years 1995-2001. The three time periods defined for this study are indicated by vertical lines.

The frequency with which an SCA was identified as a function of the number of diagnostic tests performed is illustrated in Figures 4 and 5. Statistical analysis indicated that the detection rates increased throughout the study period, with a polynomial distribution representing the best fit. The data correlated with changes in clinical practice during the three defined time periods (1995-2003, 2004-2015, 2016-2021): detection rates increased at the beginning, remained stable between 2004 and 2015, before increasing again after 2015. Figure 6 illustrates the frequency of prenatal diagnosis of the combined SCAs, 45, X, and combined SCTs as a function of the number of diagnostic procedures and live births. The highest rates of detection were in the third period. The indications for prenatal diagnostic testing that resulted in a diagnosis of any SCA, 45, X, or any SCT are shown in Table 1. Maternal age was the most common indication for diagnostic testing in the first and second time periods for the SCAs combined. During the third period NT/CH/ hydrops, maternal age, and cfDNA predominated. For 45, X specifically, NT/CH/ hydrops was the indication for most prenatal diagnostic tests. For the SCTs, maternal age was the
predominant indication for the first two time periods, with cfDNA responsible for 31.4% in the third period.

There was a positive correlation between a diagnosis of 45, X with NT/CH/hydrops and a diagnosis of SCTs and AMA across the three time periods. Spearman correlation revealed a significant, positive correlation between time period and cfDNA for all SCAs (r(4)=0.96, P=0.007).
DISCUSSION

Our study represents a population-based depiction of the relationship between changes in genetic testing practices and the prenatal diagnosis of SCAs over 27 years. Although birth rates and the frequency of diagnostic prenatal testing have declined, the frequency with which 45, X and the SCTs are identified, specifically 47, XXY, has significantly increased. There has been a reduction in tests performed solely for AMA and an increase in those due to screening test results of FCT and cfDNA. A high-risk cfDNA result was the indication for almost 31.4% of the detected SCAs between 2016-2021.

Similar to Howard-Bath et al, as a reduction in the frequency of invasive prenatal testing occurred, there was an increase in the detection rate of SCAs as function of the noninvasive testing. Our study also reveals that with the introduction of cfDNA, the diagnosis rate for SCTs and 45, X as a function of the number of live births increased significantly. The characteristics of a condition eligible for genetic screening have been clearly defined based on these stipulated characteristics. The frequency of the SCAs, particularly the SCTs support their inclusion in screenings, however, professional society guidelines have not universally endorsed the incorporation of SCAs in cfDNA screening because of (i) the high incidence of fetoplacental mosaicism, especially for 45, X, (ii) the variability of phenotype associated with SCAs and (iii) the controversy regarding the magnitude of the impacts of early intervention for a variety of associated phenotypic differences. In 2022, the evidence-based review by the American College of Medical Genetics and Genomics (ACMG) acknowledged that cfDNA is the only laboratory-based prenatal screening that identifies SCAs effectively.

High risk cases of 45, X without ultrasound scan abnormalities present a challenge to genetic counseling and clinical management because they are complicated by the increased chance of...
detecting a mosaic 45, X and sex chromosome structural rearrangement on amniocytes. In the 2015 ISPD position statement the inclusion of the sex chromosomes in cfDNA screening tests is discussed and a framework for care provided. An expanded discussion on the SCA are provided in a publication by Dondrop et al. The clinical utility afforded by the identification of individuals with SCAs using genetic screening remains a source of controversy. The definition of clinical utility is multi-faceted and should not be constrained to an evaluation of whether the performance of screening for SCAs reduces the rate of livebirths. There is compelling evidence to support the diagnosis of SCA using cfDNA to allow for targeted interventions as multiple studies have documented the improved outcomes for individuals with both 45, X and the various SCTs when they are ascertained early.

The phenotypic differences observed in individuals with 45, X are highly dependent upon the method and timing of ascertainment. Prenatal ascertainment has been primarily limited to those with serious structural cardiac defects and lymphedema. Women with 45, X may be diagnosed later due to phenotypic differences which are amenable to modification when identified early including: reduced height, pubertal delay, recurrent pregnancy loss and infertility.

The deleterious effects of delay in diagnosis for individuals with SCTs are well documented. The potential for early intervention to modify the neurodevelopmental and behavioral phenotype is well described and continues to broaden for SCTs. SCTs diagnosed prenatally had less neurodevelopmental dysfunction, higher intellectual abilities, and improved outcomes. Prenatal diagnosis not only removes the diagnostic odyssey, but allows for early intervention and treatment to address delayed puberty, infertility, potential gynecomastia, and behavioral symptoms. Specifically, the prenatal diagnosis of 47, XXY allows for the discussion of early hormonal treatment of testosterone (EHT) during mini-puberty. Numerous research
articles reveal the significant association between EHT and positive neurodevelopmental outcomes.31, 33

Prenatal screening and diagnosis provide parents the opportunity to receive appropriate counseling with facilitating informed decision-making. According to a survey-based study in 2019, 88.1% of parents recorded that a prenatal diagnosis positively influenced their child’s life.42 Benefits of this early ascertainment included the opportunity to learn more about the disorder prior to birth (35.3%), coordinate resources and interventions (38.2%), and prepare for potential neurodevelopmental delays (20.6%).42

The attitudes of individuals and families with these conditions should be considered when guidelines are developed regarding inclusion of the SCAs in the general population cfDNA screening.43 In a parent survey of children with SCAs, the majority supported prenatal screening that includes SCAs.42 Parents of children with SCA have expressed the need for healthcare providers to be better informed about the care of these disorders. Inclusion of the SCAs expands the information needed for pre-screening and counseling for expecting parents.42 Counseling and providing families with information about SCAs may be difficult for providers who are not familiar with SCAs. The ACMG practice guidelines recommend that counseling about SCAs should be based on prospective follow-up of children born following a prenatal diagnosis of that specific SCA.24 The present study has some limitations, including the absence of data regarding pregnancy management and outcomes of SCA pregnancies. These results represent a single laboratory experience in a specific geographic location. Availability of pretest and post-test screening for cfDNA screening that includes SCAs may vary due to differences in healthcare systems, access to healthcare providers with genetic expertise, and public policy/professional society guidelines. Indications for invasive procedures have been assigned by healthcare providers
located at numerous clinical centers which may be a source of some variability. However, the 27-
year longitudinal study and consistent reporting practices allowed the evaluation of both the
frequency of abnormal prenatal diagnoses and the knowledge of the indications for diagnostic
testing over time.

This comprehensive study reveals that the rate of detection per invasive test of SCTs
increased with the introduction of cfDNA testing. This has practical implications for prenatal
counseling, and medical practitioners should be cognizant of Early Hormonal Treatment (EHT)
for infants with 47, XXY as a possible means to mitigate some aspects of the neurodevelopmental
dysfunction. Our study demonstrates that increased use of cfDNA will result in more individuals
with SCAs coming to medical attention prenatally. Early interventions are available to maximize
the long-term health and well-being of individuals with SCAs, and they satisfy
requirements for consideration in population-based screening programs. Neurodevelopmental
differences that are commonly associated with SCAs may be improved with early intervention.
For example, the neuromotor profile of boys with 47, XXY includes positional torticollis
secondary to decreased muscle tonus and often limited “tummy time” exposure. This requires
timely referral to pediatric physical therapy in order to prevent and/or minimize plagiocephaly.

Early detection of 47, XXY gives parents adequate time to facilitate a visit to appropriate pediatric
specialists for the management of torticollis and prevention of secondary symptoms.

Offering cfDNA screening including the SCAs as the primary approach for all pregnancies
provides an opportunity to optimize outcomes for these individuals and their families. We support
efforts to provide the ever-expanding knowledge base to obstetrical care providers, enabling
evidence-based research and guideline development regarding offering and counseling cfDNA
screening for SCAs.
ACKNOWLEDGEMENTS

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Disclosure

FRG is currently full-time employee of R&D Menarini Biomarkers Singapore. FM, BG, RL, SM, SC, AT, CA, ER are full-time employees of TOMA Advanced Biomedical Assays S.p.A. (Impact Lab) without ownership shares.
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FIGURE LEGENDS

Figure 1: Number of CV and AF tests performed over years 1995 to 2021 per number of live births. The blue-dashed lines indicate three defined periods (1995-2003, 2004-2015, 2016-2021).

Figure 2: Number of CV tests performed over years 1995 to 2021 per number of live births. The orange-dashed lines indicate three defined periods (1995-2003, 2004-2015, 2016-2021).

Figure 3: Number of AF tests performed over years 1995 to 2021 per number of live births. The orange-dashed lines indicate three defined periods (1995-2003, 2004-2015, 2016-2021).

Figure 4: Total Detection Rate over the years 1995 to 2021 for CVS with a linear trend (a) and polynomial trend (b).

Figure 5: Total Detection Rate over the years 1995-2021 for AF with a linear trend (a) and polynomial trend (b).

Figure 6: Frequency of prenatal diagnosis of combined SCAs (a,b), 45, X (c,d) and combined SCTs (e,f) as a function of the number of diagnostic procedures (a,c,e) and live births (b,d,f).

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*sex chromosome trisomies (SCT); advanced maternal age (≥35y; AMA); anxiety (<35y); cell-free DNA (high risk or inconclusive; cfDNA); increased nuchal translucency (NT), cystic hygroma (CH) and hydrops; ultrasound scan abnormality excluding NT, CH, hydrops (US anomaly); increased or intermediate risk with another traditional screening method combining NT and serum analytes; intrauterine fetal death (IUFD); Other (e.g.: positive family history, parent carrier of a balanced chromosome abnormality, risk for monogenic disorder, IVF pregnancy, infectious disease, indication not specified
Figure 6: Trends in the rate per invasive test and rate in population over time for different genetic conditions.

- **a) Rate per invasive test**
  - CV
  - Amnio
  - CV/Amnio

- **b) Rate in population**

- **c) Rate per invasive test**
  - MX (45,X)

- **d) Rate in population**

- **e) Rate per invasive test**
  - SCT (XXX, XXY, XYY)

- **f) Rate in population**