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Clinical validity and utility of preconception expanded carrier screening for the management of reproductive genetic risk in IVF and general population

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STUDY QUESTION: What is the clinical validity and utility of preconception Expanded Carrier Screening (ECS) application on the management of prospective parents?

SUMMARY ANSWER: The high detection rate of at-risk couples (ARCs) and the high proportion opting for IVF/preimplantation genetic testing (PGT) treatment demonstrate the clinical utility of ECS in the preconception space in IVF and general population.

WHAT IS KNOWN ALREADY: About 2–4% of couples are at risk of conceiving a child with an autosomal recessive or X-linked genetic disorder. In recent years, the increasing cost-effectiveness of genetic diagnostic techniques has allowed the creation of ECS panels for the simultaneous detection of multiple recessive disorders. Comprehensive preconception genetic screening holds the potential to significantly improve couple's genetic risk assessment and reproductive planning to avoid detectable inheritable genetic offspring.

STUDY DESIGN, SIZE, DURATION: A total of 3877 individuals without a family history of genetic conditions were analyzed between January 2017 and January 2020. Of the enrolled individuals, 1212 were gamete donors and 2665 were patients planning on conceiving from both the IVF and the natural conception group. From the non-donor cohort, 1133 were analyzed as individual patients, while the remaining ones were analyzed as couples, for a total of 766 couples.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A focused ECS panel was developed following American College of Obstetrics and Gynecology ACOG-recommended criteria (prevalence, carrier rate, severity), including highly penetrant severe childhood conditions. Couples were defined at-risk when both partners carried an autosomal recessive pathogenic/likely pathogenic variant (PLP) on the same gene or when the woman was a carrier of an X-linked PLP variant. ARC detection rate defined the clinical validity of the ECS approach. Clinical utility was evaluated by monitoring ARCs reproductive decision making.

MAIN RESULTS AND THE ROLE OF CHANCE: A total of 402 individuals (10.4%) showed PLP for at least one of the genes tested. Among the 766 couples tested, 173 showed one carrier partner (22.6%), whereas 20 couples (2.6%) were found to be at increased risk. Interestingly, one ARC was identified as a result of cascade testing in the extended family of an individual carrying a pathogenic variant on the Survival Of Motor Neuron 1*SMN1* gene. Of the identified ARCs, 5 (0.7%) were at risk for cystic fibrosis, 5 (0.7%) for fragile X syndrome, 4 (0.5%) for spinal muscular atrophy, 4 (0.5%) for Beta-Thalassemia/Sickle Cell Anemia, 1 (0.1%) for Smith-Lemli-Opitz Syndrome and 1 (0.1%) for Duchenne/Becker Dystrophy. Fifteen ARCs were successfully followed up from both the IVF and the natural conception groups. All of these (15/15) modified their reproductive planning by undergoing ART with Preimplantation Genetic Testing for Monogenic

disease and Aneuploidies (PGT-M and PGT-A). To date, 6/15 (40%) couples completed their PGT cycle with euploid/unaffected embryos achieving a pregnancy after embryo transfer and three of them have already had an unaffected baby.

LIMITATIONS, REASONS FOR CAUTION: The use of a limited panel of core gene-disease pairs represents a limitation on the research perspective as it can underestimate the rate of detectable carriers and ARCs in this cohort of prospective parents. Expanding the scope of ECS to a larger panel of conditions is becoming increasingly feasible, thanks to a persistent technological evolution and progressive cataloging of gene–disease associations.

WIDER IMPLICATIONS OF THE FINDINGS: These results highlight the potential clinical validity and utility of ECS in reducing the risk of a pregnancy affected by a detectable inheritable genetic condition. The steady reduction in the costs of genetic analyses enables the expansion of monogenic testing/screening applications at the preimplantation stage, thus, providing valid decisional support and reproductive autonomy to patients, particularly in the context of IVF.

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Introduction

About 2–4% of reproductive couples are at risk of conceiving a child with an autosomal recessive (AR) or X-linked (XL) genetic disorder (Bell et al., 2011; Henneman *et al.*, 2016; Nguengang Wakap *et al.*, 2020). Although genetic diseases are individually rare, they become fairly common when considered cumulatively (Capalbo *et al.*, 2019). Couples are defined as 'at-risk' (ARC) when both partners carry an AR pathogenic/likely pathogenic variant (PLP) on the same gene or when the female partner is a carrier of an XL condition.

Carrier screening can detect whether prospective parents are at increased risk to transmit a genetic disorder to their offspring. Once identified, counseling and further information can be offered to provide a personalized reproductive planning, thus allowing for increased reproductive autonomy and informed decision making (Achterbergh *et al.*, 2007; Human Genetics Commission, 2011; Dondorp et al., 2014; 'Committee Opinion No. 691 Summary: Carrier Screening for Genetic Conditions', 2017; Delatycki *et al.*, 2020). Carrier status of a fetus can be assessed during pregnancy through prenatal tests (i.e. amniocentesis, Chorionic villus sampling, CVS). However, this approach leaves limited alternatives in the case the fetus is found affected by a genetic condition. Differently, when patients' carriership is tested at the preconception stage, identified ARCs have multiple reproductive options to choose from including IVF with preimplantation genetic test (PGT), use of donor gametes, prenatal diagnostic testing, adoption, as well as the informed choice of taking no further action. Interestingly, carrier screening (CS) programs routinely testing for cystic fibrosis (CF), fragile-X syndrome (FXS) and spinal muscular atrophy (SMA) at the preconception and early pregnancy stages show that these three conditions provide a combined risk comparable to the one of Down's syndrome, for which screening is widely accepted as part of pregnancy care (Archibald *et al.*, 2018) and often more expensive than CS.

Scientific societies, including ACOG (American College of Obstetricians and Gynecologists) and a recent ethical reflections from the ESHRE Ethics and Law, recognize that the extension of preconception genetic screening tests for common and severe recessive conditions to healthy individuals without family history is an acceptable strategy and crucially beneficial, provided that proportionality criteria of the testing strategy are met (Human Genetics Commission, 2011; Edwards *et al.*, 2015; Henneman *et al.*, 2016; Ben-Shachar *et al.*, 2019; Genomics Advisory Working Group & Women's Health Committee, 2019; de Wert *et al.*, 2021).

Despite being recognized as an effective strategy to avoid the transmission of most inheritable disorders, expanded carrier screening (ECS) is not yet routinely offered for preconception/pregnancy risk assessment. This is probably due to inconsistency with good practice recommendations in its early application (Stevens *et al.*, 2017; Chokoshvili *et al.*, 2018), combined with some persisting technical issue (such as the imperfect accuracy of genomic variants interpretation) (Richards *et al.*, 2015; Ellard et al., 2020) and poor educational programs on basic genetics for patients and the broader medical community. Before being applied to the general population, ECS can be easily introduced into the IVF process, incorporating it in the ordinary patients' pre-treatment work-up. IVF patients face a highly medicalized reproductive process which is minimally affected by the inclusion of an additional test. Moreover, couples seeking infertility treatments are generally keen on receiving additional information on their reproductive risks and, in the case increased genetic risk is detected, modest modifications to their reproductive treatment would be required (i.e. preimplantation genetic testing for monogenic diseases, PGT-M). Here, we show how even a focused ECS approach targeted to pathogenetic variants producing severe, highly penetrant recessive conditions with childhood onset has the potential to detect a significant proportion of ARCs. This ECS panel has been clinically applied on a large population of prospective parents and in gamete donor programs providing relevant information about its technical and clinical validity, as well as its clinical utility (Watson, 2015) for both directly tested individuals and for cascade-tested first-degree relatives.

Materials and methods

Study population and design

This study includes ECS results from 3877 individuals of mostly Caucasian origin without a family history of genetic conditions. In particular, 3029 individuals performed ECS by quantitative PCR (qPCR) (ECS-1) and 848 ECS by next-generation sequencing (NGS) (ECS-2).

The study population included individuals planning to conceive naturally (recruited from obstetrics and gynecology general practices) or through *in vitro* fertilization and gamete donors attending reproductive clinics affiliated with Igenomix Italy between January 2017 and January 2020. ARC detection rate was used to define the clinical validity of the ECS approach. Clinical utility was evaluated through direct monitoring of reproductive decisions made by the identified ARCs, with a minimal follow-up of 12 months. For ARCs undergoing PGT-M and preimplantation genetic testing for aneuploidies (PGT-A), IVF, and genetic procedures were performed as previously described and according to standard clinical practices (Capalbo *et al.*, 2016). Briefly, qPCR based on the use of Taq-Man allelic discrimination assays (ThermoFisher Scientific, Waltham, MA, USA) was used for direct mutation and indirect linked marker PGT-M analysis as detailed by Zimmerman *et al.* (2016). PGT-A was performed on unaffected embryos using a qPCR-based protocol consisting of 96 different TaqMan Copy Number Assays extensively validated for whole chromosome copy number aneuploidies detection on trophectoderm TE samples (Treff *et al.*, 2013; Capalbo *et al.*, 2015).

The absence of a family history of inheritable genetic disorders was ascertained through a specific questionnaire and during counseling sessions at the time of signing consent forms.

Of all the enrolled individuals, 1892 were from couples (1396 undergoing IVF and 496 natural conception) and 1212 were gamete donors (67 males and 1145 females). In addition, in accordance with a *one-member screening strategy*, a sequential approach in which one member is initially screened and a follow-up analysis is performed on the partner depending on the carrier status detected, 283 individuals undergoing homologous IVF, 794 heterologous IVF and 56 natural conceptions were analyzed. Data were collected and elaborated anonymously following Institutional Review Board approval.

Disease selection and gene panel design

The disorders were assessed for their prevalence, carrier rate and severity in terms of intellectual disability and life expectancy (Capalbo *et al.*, 2019). The selection of monogenic diseases to include in the testing panel followed the criteria recommended by the American College of Medical Genetics and Genomics (ACMG) and ACOG, including considerations regarding single nucleotide variants SNV pathogenicity (including only ACMG PLPs) and degree of gene-disease association (Lazarin *et al.*, 2014; Rehm *et al.*, 2015; Ceyhan-Birsoy *et al.*, 2017). In 2015, ACMG and the Association for Molecular Pathology (AMP) published guidelines for the interpretation of sequence variants (Richards *et al.*, 2015). The framework proposed classifies variants as 'pathogenic', 'likely pathogenic', 'of uncertain significance', 'likely benign' or 'benign'. The document also recommends that each identification of PLP conditions should be reported in terms of name of the variant, classification, associated disease and inheritance pattern, while variants of uncertain significance (VUS) should not be used in clinical decision making. Accordingly, the general consensus in today's ECS clinical practice is to report only PLP variants (Genomics Advisory Working Group & Women's Health Committee, 2019; de Wert *et al.*, 2021), which are defined as having a posterior probability of being disease-causing of >99% and >90%, respectively (Richards *et al.*, 2015). As per standard diagnostic practice, variants annotation was updated at regular intervals over the study period. Ten recessive disorders were included in ECS-1: *SMA* (OMIM #253300); long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (*LCHCDD*, OMIM #609016); medium-chain acyl-CoA dehydrogenase deficiency (*MCACDC*, OMIM #201450); congenital disorder of glycosylation 1a (CDG1a, OMIM #212065); *CF* (OMIM #219700); metachromatic leukodystrophy (*ML*, OMIM #250100); FXS (OMIM #300624); Smith-Lemli-Opitz syndrome (*SLOS*, OMIM #270400); Duchenne and Becker Muscular Dystrophy (*DMD-DMB*, OMIM #310200- #300376). In ECS-2, 12 additional disorders were included: Glycogen Storage Disease 2 (*GSD*, OMIM #232300); Hyperoxaluria, primary, type 1 (*HP1*, OMIM #259900); Biotinidase Deficiency (*BD*, OMIM #253260); Homocystinuria due to Cystathionine beta-synthase Deficiency (*HCBD*, OMIM #236200); Beta-Thalassemia/Sickle Cell Anemia (*BT/SCA*, OMIM #613985-#603903); Galactosemia (*GAL*, OMIM #230400); Phenylketonuria (PHE, OMIM #261600); Methylmalonic aciduria and homocystinuria (*MAH*, OMIM #277400); Emery-Dreifuss Muscular Dystrophy (*EDM*, OMIM #310300); Fabry Disease (*FD*, OMIM #301500); Achondrogenesis type 1b (*ACHO1*, OMIM #600972).

Gene-disease associations are described in Supplementary Table SI. Classification and interpretation of variants were performed according to guidelines recommended by ACMG (Richards *et al.*, 2015) and using public databases (ClinVar, Varsome, dbSNP, CFTR2). The cluster of all PLP variants considered in this approach is reported in Supplementary Table SII. In the clinical workflow, patients were counseled about the imperfect accuracy of variants classification, and that only PLP variants were reported as per standard practice. Residual risks were provided according to the specific carrier profile of each couple. Meaningful residual risk calculation is particularly effective for focused ECS panels like the one used here, where the most common and severe recessive conditions are included.

Individuals were identified as carriers if they were positive for at least one of the targeted PLP variants. Couples were classified as ARC if both male and female partners were carriers for the same AR disorder or the female partner was carrier for an XL recessive disorder.

Experimental procedures

DNA samples were extracted from blood using PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Rodano, Italy) and DNA concentrations were measured using Nanodrop (Thermo Fisher Scientific). The ECS test was conducted in multiple steps:

Genotyping assays (ECS-1)

All single nucleotide polymorphisms or the small insertion deletion (SNPs/indel) assays were designed by Thermo Fisher and pre-spotted in 384-well plates. Genotyping assay was performed following manufacture's instruction using 12.5 ng of DNA mixed with the TaqMan™ Gene Expression Master Mix (Catalogue number: 4369016, Thermo Fisher Scientific). The allelic specificity of TaqMan assay was provided by two probes, one labelled with carboxyfluorescein (FAM) dye and the other with VIC dye. Rox was used as a passive reference dye.

Melting curve analysis (FXS) (ECS-1 and ECS-2)

FXS carrier screening was performed by triplet-primed polymerase chain reaction of the fragile × mental retardation 1 (FMR1) CGG repeat region using 3' direct triplet-primed PCR and melting curve analysis. The 3'dTP-PCR were set up as described in (Teo *et al.*, 2012; Lim *et al.*, 2015). Melting curve profiles were generated by plotting

$-dF/dT$ (negative first derivative of fluorescence over temperature) against T (temperature) using two reference DNAs with known CGG repeats. Baseline temperature was selected to discriminate Normal repeats (≤54) from Grey Zone/Pre-Mutation (≥55) and Full Mutation (≥200). All samples from individuals with increased risk results had confirmatory testing through standard diagnostic testing protocols using Triplet-primed PCR and DNA Fragment Analysis by Capillary Electrophoresis. The results were consistently concordant.

Copy number variation analysis (SMA and DMD) (ECS-1 and ECS-2)

A quantitative real-time PCR was used to measure the Copy Number (CN) of exon 7 of the Survival Of Motor Neuron 1 (*SMN1*) gene and intron 6 and exons 13, 46, 50 of the *DMD* gene. The presence of a deleted allele and two *cis* copies of the gene on the other allele could not be detected. TaqMan Copy Number Reference Assay RNase P (Catalogue number: 4403326, Thermo Fisher Scientific) was used as standard reference assay for CN.

Library preparation and sequencing (ECS-2)

Ion Ampliseq™ Designer was used to generate a multiplex PCR amplification strategy for the coding sequences and 25 base pair exon padding of the target region previously specified (Supplementary Table SIII). The panel constituted by 229 amplicons which covered over 99.3% of the target sequence (Supplementary Table SIV). A total of 2.5 ng DNA per sample was used for target enrichment by multiplex PCR and each DNA pool was amplified with the Ion Ampliseq™ Library Kit in conjunction with the Ion Ampliseq™ 'custom Primer Pool'. After amplification, each library was barcoded and purified according to the manufacturer's procedures (Life Technologies, Carlsbad, CA, USA). Equalized barcoded libraries were pooled into batches ranging between 16 and 80 samples at a time. The number of combined libraries that could be accommodated in a single sequencing run depended on the size of the chip, the balance of barcoded library concentration and the coverage required. Pools at 33 pM concentration were loaded on Ion Chef ™ Instrument (Life Technologies, Carlsbad, CA, USA) for template enrichment. Sequencing was performed using a commercially available sequencing kit (Ion S5 XL System; Life Technologies, Milano, Italy) and as per the manufacturer's instructions.

Torrent Suite™ software was used to compare base calls, read alignments, and variant calling with the human genomic sequence reference (hg19). The variants selected for further analysis met the following criteria: (a) the variant was detected in sequence reads for both strands, (b) a minimum coverage of 20× was achieved and (c) the variant heterozygosity ranged between 35% and 65%.

Technical validity: protocol validation on positive controls

The technical validity of the methodologies employed for ECS testing was evaluated on both positive and negative controls. Validation was based on the assessment of 198 samples with known genotypes, as characterized by independent techniques. SNVs and copy number variant CNVs tests validation were performed blinded on pre-spotted plates with positive and negative controls. For TaqMan Targeted Genotyping, 24/24 (100%, 95% CI 85.7–100) positive and 34/34 (100%, 95% CI 89.7–100) negative samples were confirmed showing a >99% sensitivity and >99% specificity. For NGS, 26/26 (100%, 95% CI 86.8–100) positive at least one variant and 16/16 (100%, 95% CI 79.4–100) negative samples for all variants were confirmed showing a >99% sensitivity and >99% specificity. Moreover, sequencing data were converted from hg19 to the hg38 alignment and Torrent Suite™ software was relaunched. The conversion procedure resulted in 2207/2207 detected variants (100%, 95% CI 99.8–100) and 100 randomly selected positive individuals were confirmed with medium coverage of 900× and variant heterozygosity of 50.9%±2.3% per base call.

For Melting Curve Analysis (FXS), 24/24 (100%, 95% CI 85.7–100) pre/full-mutated controls and 26/26 (100%, 95% CI 86.8–100) normal controls were confirmed. Moreover, all detected pre-mutated patients were successfully confirmed using Triplet-primed PCR and DNA Fragment Analysis by Capillary Electrophoresis. For CNV Analysis, 12/12 (100%, 95% CI 73.5–100) positive controls (8 for SMA, 4 for DMD) and 36/36 (100%; 95% CI 90.3–100) negative controls were detected. Based on the robustness of the assays validation results (>99% sensitivity and a >99% specificity), these ECS tests were employed for subsequent diagnostic routine.

Statistical analysis

Data are expressed as mean ± standard deviation or percentages and 95% CI as appropriate. Proportions were compared using Chi-squared test or Fisher's exact test. In the analytical validation phase, the diagnostic sensitivity and specificity of each methodology was measured on a per sample level as the proportion of actual positive genomic variants that were consistently identified as such, and the proportion of consistently identified negatives. All tests were two-tailed. All analyses were conducted using SPSS v. 21 and R v. 3.5.1 (SPSS Inc., Chicago, IL, USA).

Results

Clinical validity: disease carrier frequencies and at-risk couples' rate

The carrier frequencies of the conditions analyzed in the ECS are presented in Table I and Fig. 1A–C. Of the 3877 screened individuals, 10.4% (402/3877) were found to be carriers of at least one condition. Of these, 366 individuals showed only one PLP, whereas 33 individuals were positive for 2 variants, 2 individuals for 3 variants, and 1 for 4 variants (Fig. 1B). Among all 1212 gamete donors, 11/67 (16.4%) males were identified as carriers of an AR condition. In female gamete donors 97/1145 (8.5%) were identified as carriers of an AR disease, and 5/1145 (0.4%) as carriers of an XL recessive (XLR) condition.

The four most common diseases were Phenylketonuria, Beta-Thalassemia/Sickle Cell Anemia, CF, SMA with an observed carrier risk (CR) of 4.4%, 3.2%, 2.9% and 2.3%, respectively (Table I). Of note, in our gene-disease panel, we found PLP variants in the phenylalanine-hydroxylase (PAH) gene of 4.4% of individuals, ranking as the most common condition in our analysis. Phenylketonuria (PKU), caused by variants in the PAH gene, is the most common autosomal-recessive Mendelian phenotype of amino acid metabolism in general and our observed carrier rate compared well with known prevalence of the conditions in our region. Indeed, PKU is found more commonly in Caucasian populations, with an approximate incidence of 1:10 000

Table I Carrier frequencies of gene/diseases included in expanded carrier screening (ECS) detected in our cohort of Caucasian individuals.

Disease	Gene	Carrier frequency		
		N	%	1 in
Phenylketonuria	*PAH*	37/848	4.4%	1 in 23
Beta-thalassemia/sickle cell anemia	*HBB*	27/848	3.2%	1 in 31
Cystic fibrosis	*CFTR*	113/3,877	2.9%	1 in 35
Spinal muscular atrophy	*SMN1*	88/3,877	2.3%	1 in 44
Glycogen storage disease 2	*GAA*	12/848	1.4%	1 in 71
Methylmalonic aciduria and homocystinuria	*MMACHC*	10/848	1.2%	1 in 85
Smith-Lemli-Opitz syndrome	*DHCR7*	44/3,877	1.1%	1 in 88
Congenital disorder of glycosylation 1a	*PMM2*	41/3,877	1.1%	1 in 95
Achondrogenesis type 1 b	*SLC26A2*	8/848	0.9%	1 in 106
Homocystinuria due to cystathionine beta-synthase deficiency	*CBS*	6/848	0.7%	1 in 141
Medium chain acyl-CoA dehydrogenase deficiency	*ACADM*	24/3,877	0.6%	1 in 162
Biotinidase deficiency	*BTD*	4/848	0.5%	1 in 212
Fragile-X syndrome	*FMR1*	10/2,190	0.5%	1 in 219
Galactosemia	*GALT*	3/848	0.4%	1 in 283
Hyperoxaluria, primary, type 1	*AGXT*	2/848	0.2%	1 in 424
Long chain 3-hydroxyacyl-CoA dehydrogenase deficiency	*HADHA*	8/3,877	0.2%	1 in 485
Metachromatic leukodystrophy	*ARSA*	6/3,877	0.2%	1 in 646
Duchenne/Becker muscular dystrophy	*DMD*	1/2,190	0.05%	1 in 2,190

and, therefore, a carrier frequency of around 2% in Caucasians (Hardelid *et al.*, 2008). More recently, a global analysis of prevalence of this condition among different ethnicities and countries worldwide revealed that Italy ranked as the country with the highest PKU prevalence (1:4000) supporting our direct genetic screening experience (Hillert *et al.*, 2020).

Distribution of the different variants for each pathology is reported in Fig. 1C and Supplementary Table SV. The three most commonly detected variants were exon 7 deletion (*SMN1*), c.1521_1523delCTT (CF transmembrane conductance regulator, *CFTR*) and c.118C>T (Hemoglobin Subunit Beta, *HBB*). Notably, we reported an exceptionally high frequency of splicing variant IVS8-5T (only ECS-1), present in 189/3,029 individuals (6.2%), whereas the IVS8-TG test was performed only on IVS8-5T positive patients in couples where the partner was positive for one of the pathogenic variants in *CFTR* gene.

Among the 766 couples tested, 173 (22.6%) showed one carrier partner and 19 (2.5%) were found to be ARCs. Notably, four of these were identified among couples trying to conceive by natural conception. CF and FXS were the most common diseases in carrier couples (n = 5, 0.7% each), followed by SMA (n = 4, 0.5%), HBB (n = 4, 0.5%), SLOS (n = 1, 0.1%) and DMD-DMB (n = 1, 0.1%) (Fig. 2A). Remarkably, the identification of a patient carrying a pathogenic variant on the *SMN1* gene led to cascade testing of his brother, revealing an additional ARC for SMA (Fig. 2C). In total, 20 ARCs were identified in this ECS experience on 766 couples (2.6%).

Moreover, two different carriers of two pathogenic variants each were detected (c.14C>T, *CFTR* and c.2491G>T, *CFTR* in one patient whereas c.734T>C, *PAH* and exon 7 deletion, *SMN1* in another one) and ECS as cascade testing was performed on their respective siblings. In the first case, the sister was a carrier of 2491 G > T CFTR, also showing that the two variants in *CFTR* gene were in trans configuration. In the second case, the brother was a carrier only of exon 7 deletion in *SMN1*. At the time of writing, the partners of these carrier individuals identified by cascade testing were not yet available for analysis (Fig. 2D).

Clinical utility of ECS in couples seeking to conceive spontaneously or through IVF

The clinical utility of ECS was measured by determining how many ARCs modified their reproductive decisions and pregnancy management after becoming aware of their risk status. Couples received genetic counseling and were advised on all possible reproductive options for disease risk management, including PGT-M, prenatal diagnosis (PND) and accepting the reproductive risk without any further medical/diagnostic intervention. A conclusive follow up on reproductive decision was possible in 15 out of the 20 ARCs (Table II). Among these, all of them pursued actions aimed at reducing their risk and enrolled in an IVF/PGT-M program. This was the preferred option for ARCs identified among both the IVF and the natural conception groups. At the time of writing, the other five ARCs did not show any definitive preference toward reproductive risk management. It is noteworthy that ARCs not yet opting for PGT/PND are those belonging to a lower or milder genetic risk group. Indeed, three of them were carriers of *FMR1* pre-mutation. In these cases, the fetal diseases risk for FXS is dependent on the likelihood of expansion to full mutation and in any case lower than the expected 25% for canonical recessive diseases. For these three ARCs, the pre-mutated alleles were <70 CGG repeats, with very low risk to full mutation expansion (Delatycki *et al.*, 2019). The other two couples who did not show definitive reproductive risk management preference have an increased risk for atypical CF, which associates with mild dysfunction in one organ system and might or might not have elevated sweat chloride levels. Accordingly, the lower disease risk rate and the milder expected phenotype might have driven different considerations for these ARCs. To date, 6/15 (40%) couples completed their PGT-M cycle with euploid/healthy embryos achieving a healthy pregnancy after embryo transfer. Three of them have already delivered an unaffected, healthy child (Fig. 2B).

Discussion

Despite the limited number of pathogenic conditions tested in this preliminary experience of preconception ECS, 2.5% of couples tested were identified as at high risk of conceiving an affected child. This

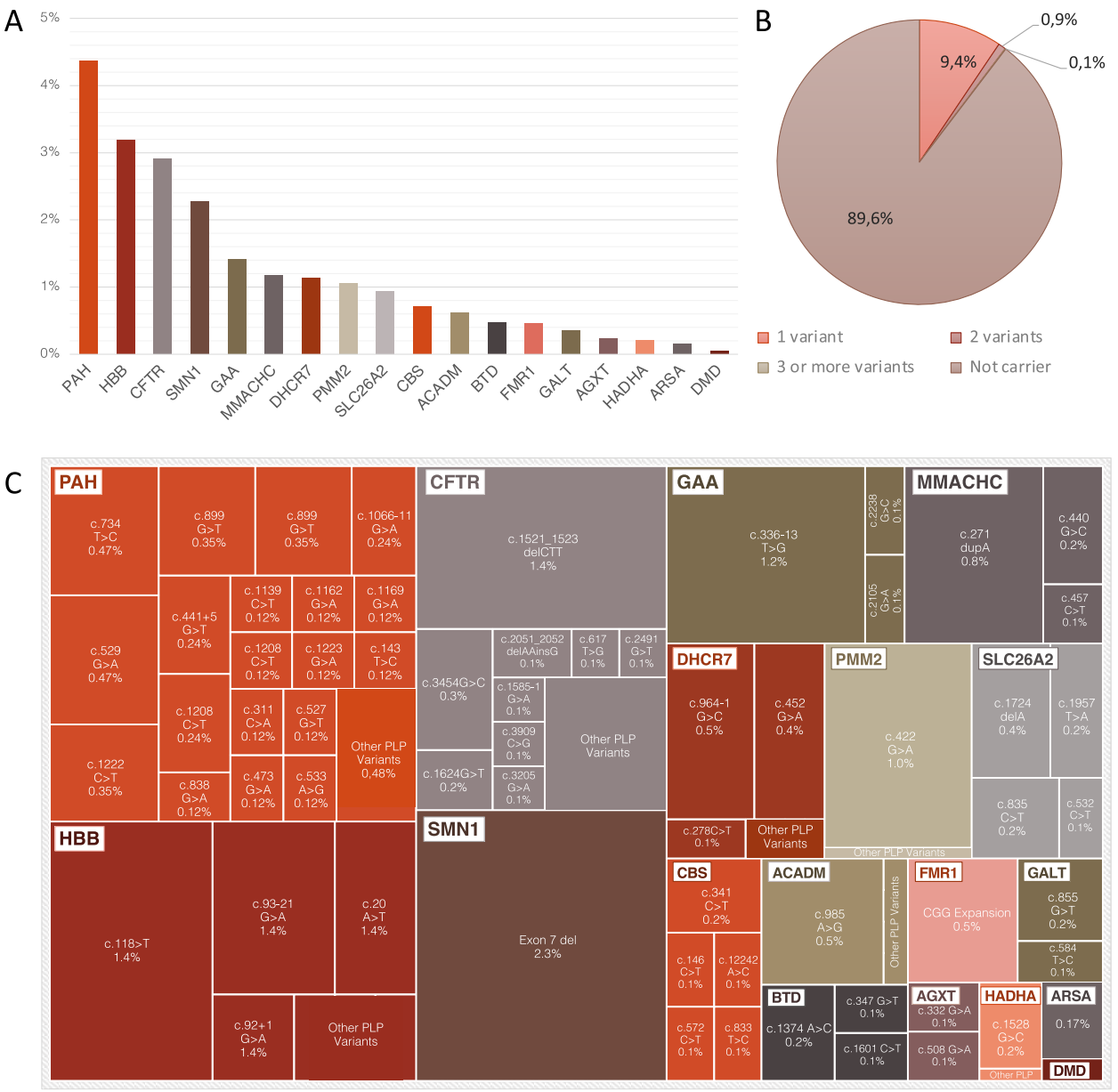

Figure 1. Detected variants details. (**A**) Percentage of identified pathogenic variants by ECS on total identified carrier for each condition; (**B**) distribution of the number of pathogenic/likely pathogenic (PLP) variants detected per individual sample; (**C**) percentage of identified variants for each gene by ECS versions 1 and 2 (Updated) on total analyzed individuals. GAA, glucosidase alpha, acid; MMACHC, Metabolism Of Cobalamin Associated C; DHCR7, 7-dehydrocholesterol reductase; PMM2, Phosphomannomutase 2; SLC26A2, Solute Carrier Family 26 Member 2; CBS, Cystathionine Beta-Synthase; ACADM, acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain; BTD, amidohydrolase biotinidase; FMR1, fragile X mental retardation 1; GALT, Galactose-1-Phosphate Uridylyltransferase; AGXT, Alanine–Glyoxylate and Serine–Pyruvate Aminotransferase; HADHA, Hydroxyacyl-CoA Dehydrogenase Trifunctional Multienzyme Complex Subunit Alpha; ARSA, Arylsulfatase A; DMD, Dystrophin.

relatively high incidence demonstrates that ECS has clinical validity even when few expertly selected gene–diseases pairs are investigated in a patient population with a negative family history of recessive genetic conditions. This rate further increased to 2.6% when positive matches in the primary population were employed to identify additional ARCs through passive cascade testing of relatives. Notably, in our longitudinal analysis, all the identified ARCs that took a definitive decision on their reproductive risk management, pursued PGT-M to avoid the risk of an affected child.

Our study has important implications for the development of general health policies for genetic risk assessment in prospective parents. In particular, several severe recessive conditions, such as CF, SMA,

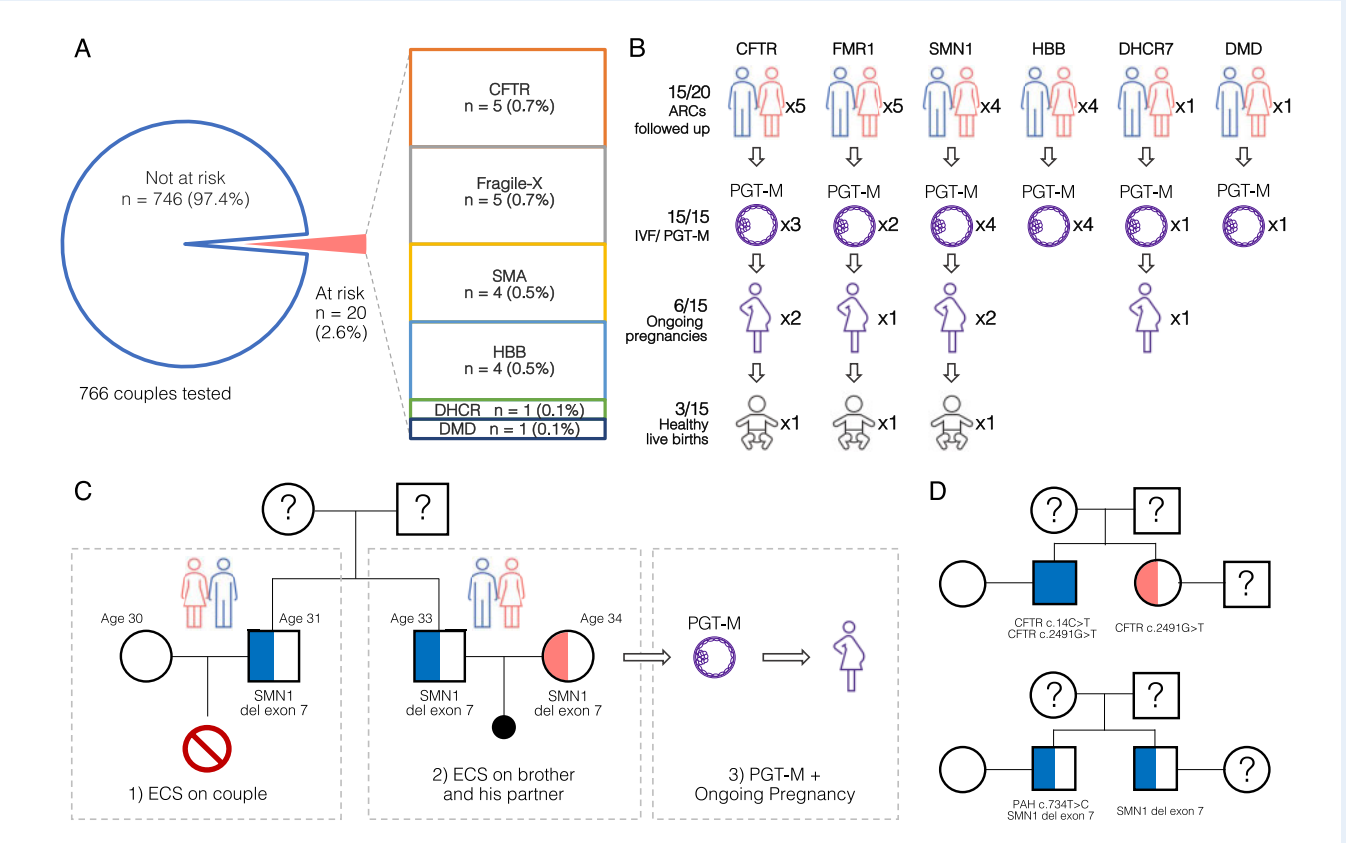

Figure 2. Evaluation of preimplantation ECS' clinical utility on the study population. (**A**) Incidence of at risk couple (ARC) detection and details on the 'at-risk' gene; (**B**) details on clinical follow up of identified ARCs; (**C**) clinical details of a cascade testing leading to identification of an additional ARC in the family, which was followed by IVF/preimplantation genetic testing for monogenic diseases (PGT-M) and resulted in a currently ongoing pregnancy; (**D**) details of two cases of cascade testing leading to the identification of additional carriers in the family.

FXS, HBB, PAH showed similar carrier rates and contributed almost uniformly to the fetal disease risk in this cohort of couples and individuals. Often, only one or a fraction of these conditions is routinely investigated in prospective parents through genetic or ancillary screening protocols as defined by national or regional health care authorities. Our results suggest that, in the absence of specific medical or anamnestic indication, all the severe conditions targeted in this study are equally worth testing and none should be overlooked or bypassed. Of note, the individual level data generated in this study revealed that carrier frequencies of the main monogenic severe recessive conditions tested were similar to those detected in study populations of different ethnicities. For example, carrier frequencies for CF in our study (1 in 35) and SMA (1 in 44) were also comparable to other published carrier frequencies (Haque *et al.*, 2016; Archibald *et al.*, 2018; Capalbo *et al.*, 2019). In our study, carrier frequency for FXS was 1 in 250, whereas Haque and Archibald reported rates of 1 in 316 and 1 in 332, respectively. This small difference can either be due to chance or related to the higher frequency of infertile individuals in our dataset. Supporting findings have also been recently reported by Guo and Gregg, where 123 136 aggregated exome sequencing (ES) samples were used to leverage carrier rate frequencies across six different ancestries (Guo and Gregg, 2019). Despite the use of aggregated whole exome sequencing (WES) data prevented the collection of important information for certain common conditions undetectable via NGS (e.g. FXS and SMA), this study showed that a few critical and often overlapping conditions contribute to a large portion of fetal disease risk, regardless of ethnical background. Thus, broad application of preconception carrier screening for an isolated condition makes little or no sense in the light of current evidence and today's technological possibilities.

In addition, the ARC identification rate observed here is significantly higher than the more-widely considered risk of fetal chromosomal aneuploidies (Hook, 1981; American College of Obstetricians and Gynaecologists, 2007) (Fig. 3). This aspect is even more striking considering that, contrary to chromosomally abnormal conceptions which incidental risk increases with female age, the reproductive risk associated with inheritable recessive diseases is perpetual throughout one's lifespan, hence, potentially affecting couples at the peak of their reproductive potential (below 35 years). As observed here, 1 in 300/400 pregnancies is expected to be affected by one of the recessive

Table II Genetic details of couples identified as at risk for autosomal and X-linked recessive conditions. MIM phenotype

Partner 1 Pathogenic Variant	Partner 2 Pathogenic Variant	Ethnicity	Origin	Gene	Pathology	MIM phenotype	MIM gene	PGT-M	Ongoing pregnancy/ live Birth
c.1521_1523delCTT	c.1521_1523delCTT	Caucasian/Caucasian	Homologous-IVF	*CFTR*	Cystic fibrosis	219700	602421	Yes	Yes
c.1521_1523delCTT	Poly5T/PolyTG12	Caucasian/Caucasian	Homologous-IVF	*CFTR*	Cystic fibrosis	219700	602421		
c.1521_1523delCTT	Poly5T/PolyTG12	Caucasian/Caucasian	Homologous-IVF	*CFTR*	Cystic fibrosis	219700	602421	Yes	Yes
c.2051_2052delAAinsG	c.3209G>A	Caucasian/Caucasian	Homologous-IVF	*CFTR*	Cystic fibrosis	219700	602421	Yes	
c.1521_1523delCTT	Poly5T/PolyTG12	Caucasian/Caucasian	Homologous-IVF	*CFTR*	Cystic fibrosis	219700	602421		
31, 57	/	Caucasian	Homologous-IVF	*FMR1*	Fragile-X Syndrome	300624	309550	Yes	Yes
30, 56	/	Caucasian	Natural Conception	*FMR1*	Fragile-X Syndrome	300624	309550	Yes	
25, 60	/	Caucasian	Natural Conception	*FMR1*	Fragile-X Syndrome	300624	309550		
23, 57	/	Caucasian	Homologous-IVF	*FMR1*	Fragile-X Syndrome	300624	309550		
30, 82	/	Caucasian	Natural Conception	*FMR1*	Fragile-X Syndrome	300624	309550		
del ex7	del ex7	Caucasian/Caucasian	Homologous-IVF	*SMN1*	Spinal muscular atrophy 1	253300	600354	Yes*	Yes
del ex7	del ex7	Caucasian/Caucasian	Homologous-IVF	*SMN1*	Spinal muscular atrophy 1	253300	600354	Yes	
del ex7	del ex7	Caucasian/Caucasian	Natural Conception	*SMN1*	Spinal muscular atrophy 1	253300	600354	Yes	
del ex7	del ex7	Caucasian/Caucasian	Homologous-IVF	*SMN1*	Spinal muscular atrophy 1	253300	600354	Yes	Yes
c.93-21G>A	c.92+2T>A	Caucasian/Caucasian	Homologous-IVF	*HBB*	Beta-Thalassemia/Sickle Cell Anemia	613985/603903	141900	Yes	
c.93-21G>A	c.118C>T	Caucasian/Caucasian	Homologous-IVF	*HBB*	Beta-Thalassemia/Sickle Cell Anemia	613985/603903	141900	Yes	
c.20A>T	c.93-21G>A	Caucasian/Caucasian	Natural Conception	*HBB*	Beta-Thalassemia/Sickle Cell Anemia	613985/603903	141900	Yes	
c.118C>T	[c.316-106C>T; c.-31C>T]	Caucasian/Caucasian	Homologous-IVF	*HBB*	Beta-Thalassemia/Sickle Cell Anemia	613985/603903	141900	Yes	
c.452G>A	c.964-1G>C	Caucasian/Caucasian	Homologous-IVF	*DHCR7*	Smith-Lemli-Opitz Syndrome	270400	602858	Yes	Yes
del ex46	/	Caucasian	Homologous-IVF	*DMD*	Duchenne/Becker Muscular Dystrophy	310200/300376	300377	Yes	

*Couple detected by cascade testing; MIM, Mendelian Inheritance in Man; PGT-M, Preimplantation Genetic Testing for Monogenic diseases; HBB, Hemoglobin Subunit Beta; CFTR, Cystic fibrosis transmembrane conductance regulator; SMN1, Survival of motor neuron 1; DHCR, Dehydrocholesterol Reductase; FMR1, fragile X mental retardation 1; DMD, Dystrophin.

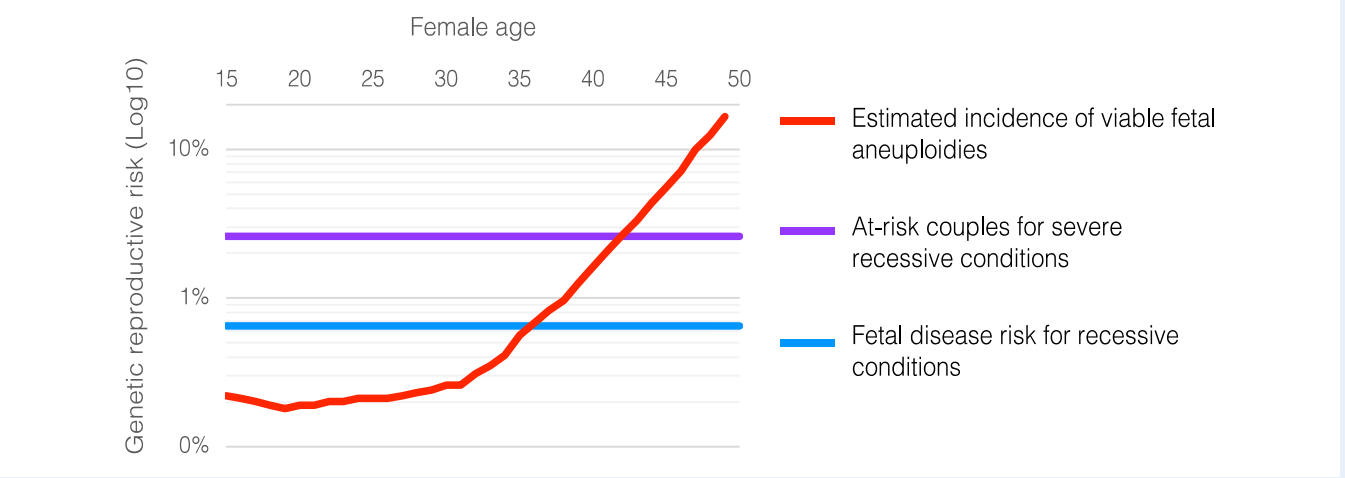

Figure 3. Association between reproductive genetic risk and female age. The detectable risk for recessive genetic conditions is constant throughout female reproductive lifespan (at-risk couples rate; purple). This risk exceeds the fetal disease risk for viable aneuploidies (red) for most of the reproductive years. Of note, the vast majority of pregnancies and births are obtained from younger women for whom recessive conditions are the main reproductive genetic risk factor (blue). Data of aneuploidies are reported from Hook (1981) and American College of Obstetricians and Gynaecologists (2007). Risk for chromosomal abnormality includes the risk for trisomy 21 and trisomy 18 in addition to trisomy 13, 47, XXY, 47, XYY, Turner syndrome genotype, and other clinically significant abnormalities, 47, XXX not included. At risk, couple rate is calculated based on the percentage of couples where both male and female partners were carriers for the same autosomal recessive disorder, or the female partner was carrier for an XL recessive disorder out of the total number of tested couples. Fetal disease risk for recessive diseases is calculated dividing the ARC detection rate by four.

conditions included in our focused ECS panel. This risk is considerably higher than the prevalence of Down's syndrome in the general population, especially for woman younger than 35 (1 in 1000). As reported here, the risk for recessive diseases is constant and often higher than the overall risk of fetal aneuploidies. Similarly, the regular application of a minimal screening approach (including CF, FXS and SMA) in preconception and prenatal programs in Australia showed a similar diagnostic yield for these three recessive conditions as compared with Down's syndrome risk (Archibald *et al.*, 2018). The high ECS diagnostic sensitivity has also been widely validated in several different ethnicities (Haque *et al.*, 2016; Guo and Gregg, 2019), suggesting the urgent need for improving the framework for education and counseling of reproductive couples in a way that is similar to what is currently done for fetal aneuploidies risk.

Importantly, among the main technical assessment criteria for genetic testing (e.g. The Accreditation Council for Continuing Education (ACCE) standards; Daly *et al.*, 2020), the clinical utility is of particular relevance and mainly depends on the availability of specific treatments or preventive measures that are able to effectively mitigate the risk. In the context of preconception ECS, clinical utility can be easily verified by monitoring ARCs' reproductive decisions. In a recent study including 64 ARCs for either a severe or a profound condition, 76% performed a PGT-M cycle or planned a prenatal diagnosis in case of successful pregnancy to reduce their risk of an affected birth (Ghiossi *et al.*, 2018). In another study performed in the IVF setting, all identified ARCs (8/8) opted for a PGT-M cycle to minimize the risk of having an affected pregnancy (Franasiak *et al.*, 2016). Our study shows similarly high clinical utility, with the majority of ARCs identified taking actions to prevent the transmission of the inheritable condition detected. Furthermore, this study documents the first report of live births of unaffected children following preconception ECS followed by IVF/PGT-M treatments.

A minority of ARCs did not pursue definitive actions in the 12 months following the ECS results. As previously shown by Johansen and colleagues, the condition's severity correlates with actionability of the findings (Johansen Taber *et al.*, 2019). Indeed, profound and severe conditions have a higher actionability rate in ARCs compared to moderate conditions. Similarly, in our experience, the five couples who did not take actions had either a lower expected disease risk due to the FMR1 pre-mutation carriership (<70 CGG repeats) or they were at risk of mild and atypical form of FC.

In addition, we showed for the first time, the clinical utility of ECS in the context of cascade analysis. In fact, one additional ARC in reproductive age was identified through cascade testing triggered by the identification of ARCs receiving preconception ECS. One of these two couples enrolled into an IVF/PGT-M program to avoid reproductive risk and an unaffected pregnancy is currently underway. As widely adopted for ancestry-based carrier screening and more recently in oncology (Super *et al.*, 1994; Marks *et al.*, 2006; Gupta *et al.*, 2019; Owens *et al.*, 2019; Daly *et al.*, 2020), targeted cascade testing is an effective strategy to screen enriched subpopulation (i.e. the family members of carrier individuals) and identify additional ARCs, as first-degree relatives have a 50% probability of having inherited the same pathogenic variant. In this context, the genetic testing can be performed by targeted variant analysis and with increased cost-effectiveness (Arroyo-Esquivel and Hastings, 2020). Of note, our cascade testing approach was passive and no direct approach for contact tracing and information of first-degree relatives of carrier individuals was

employed. Simply, primary ARCs were advised to inform first-degree relatives of reproductive age. As for other fields of genetic disease screening, novel strategies for broader implementation of cascade testing in ECS will need to be evaluated in research setting and implemented accordingly.

In this study, a limited panel of genes was used, limiting the ability to characterize the whole spectrum of severe recessive conditions detectable through ECS on a purely research setting. The selection of monogenic diseases in our test panel was restricted to the most frequent and severe ones in our target Caucasian population and followed the criteria recommended by the ACMG and ACOG, including considerations regarding a clear validated clinical association between PLP variants and disease severity (Richards *et al.*, 2015). For each selected disorder, the causative pathogenic variants and their carrier frequencies were known in the population tested, allowing residual risk calculation in individuals who tested negative (Capalbo *et al.*, 2019). The initial offer of a more focused, minimal ECS panel was preferred to a wider catalogue of genes thus to fulfill the criteria for a responsible and gradual implementation of ECS in our clinical environment (Dondorp et al., 2014; Henneman *et al.*, 2016; de Wert *et al.*, 2021). This approach is indeed compliant with the most recent ethical reflections and testing proportionality criteria mentioned in the recent publication by the ESHRE Ethics Committee (de Wert *et al.*, 2021). Expanding the scope of ECS to larger conditions panels is becoming increasingly feasible (Martin *et al.*, 2015; Kirk *et al.*, 2021), thanks to continuous technological improvements and progressive cataloguing of gene-disease associations. Recently, an effort from the Australian Reproductive Genetic Carrier Screening Project (ARGCSP; or 'Mackenzie's Mission') has been performed to compile an expanded gene-disease list worthy of being considered for ECS application. For this analysis, a multidisciplinary committee composed of clinical geneticists, genetic counselors, an ethicist, a parent of a child with a genetic condition and scientists from diagnostic and research backgrounds was created. A list of 1300 conditions was compiled using several distinct criteria for gene-disease selection,] that will be prospectively tested in 10 000 couples (Kirk *et al.*, 2021). Similar efforts are required to evaluate dynamically on a research ground the possibility of increasing the diagnostic sensitivity of ECS while balancing all the potential limitation. In this context, the imperfect accuracy of variants interpretation is a constant limitation of genomic studies and applications. It has been shown that even using similar classification schemes, variants can be interpreted differently across laboratories (Amendola *et al.*, 2016). Over time and through the accumulation of sequencing data, particularly from affected individuals, VUS re-classification in LP or likely benign (LB) will increase the accuracy of variants interpretation. For instance, a recent study by Fridman and colleagues showed that, in a population of Ashkenazi Jewish couples where one partner carried a PLP variant and the other a VUS on the same gene, if just 10% of VUS detected were to be reclassified as PLP, ECS yield would increase by ≈20% (Fridman *et al.*, 2020). It is evident that widening population frequency variant databases, particularly for affected individuals, would advance variants classification process for ECS, leading to improvements in the accuracy of ARC detection.

Furthermore, this study did not evaluate different strategies for engaging/educating prospective parents to ECS, nor did it investigate patients' attitude and preferences toward ECS, as well as the main ethical issues that the ESHRE Ethics Committee has recently and expertly addressed (de Wert *et al.*, 2021). As highlighted in this clinical experience and reviewed elsewhere (van der Hout *et al.*, 2019), the education and counseling on ECS and potential subsequent clinical management of the genetic risk is easier in IVF couples than in the general population as these couples are already undergoing medical examinations for reproductive purposes (Van Steijvoort *et al.*, 2020). Future efforts are required to understand and develop proper frameworks and strategies for application and education of individuals in their preconception years (Dugger *et al.*, 2020), with a special focus on providing equity of access for infertile couples and the general population.

In conclusion, our study shows that preconception ECS offers clinical validity and utility providing valuable support to prospective parents by improving autonomous reproductive decision making in about 3% of couples trying to conceive either through IVF or natural conception. Moreover, ECS is not expected to benefit only tested individuals, as it has shown indirect utility through cascade analysis of first-degree relatives of reproductive age. These data confirm the ongoing need for identification of barriers and tailored solutions for enabling broader adoption of preconception ECS and achieve its successful routine application, similar to fetal aneuploidy screening programs.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Authors' roles

A.C., C.S., L.R., and F.M.U. designed the study. A.C., M.F., S.C., and M.P. analyzed the data and drafted the manuscript. All other authors contributed to the collection of data and discussion of the results.

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Conflict of interest

A.C., M.F., S.C., M.P., L.G., and C.P. are employees of Igenomix Italy. C.S. is the head of the scientific board of Igenomix.

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