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Comprehensive carrier genetic test using next-generation deoxyribonucleic acid sequencing in infertile couples wishing to conceive through assisted reproductive technology

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Objective: To develop an expanded pan-ethnic preconception carrier genetic screening test for use in assisted reproductive technology (ART) patients and donors.
Design: Retrospective analysis of results obtained from 2,570 analyses.
Setting: Reproductive genetic laboratory.
Patient(s): The 2,570 samples comprised 1,170 individuals from the gamete donor programs; 1,124 individuals corresponding to the partner of the patient receiving the donated gamete; and 276 individuals from 138 couples seeking ART using their own gametes.
Intervention(s): None.
Main Outcome Measure(s): Next-generation sequencing of 549 recessive and X-linked genes involved in severe childhood phenotypes reinforced with five complementary tests covering high prevalent mutations not detected by next-generation sequencing.
Result(s): Preclinical validation included 48 DNA samples carrying known mutations for 27 genes, resulting in a sensitivity of 99%. In the clinical dataset, 2,161 samples (84%) tested positive, with an average carrier burden of 2.3 per sample. Five percent of the couples using their own gametes were found to have pathogenic variants conferring high risk for six different diseases. These high-risk couples and patients received genetic counseling and recommendations for preimplantation genetic diagnosis. For patients receiving gamete donation, we applied a genetic testing and blinded matching system to avoid high-risk combinations regardless of their carrier burden. For female donors, 1.94% were positive for X-linked conditions; they received genetic counselling and were discarded.

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Conclusion(s): We have developed a comprehensive carrier genetic screening test that, combined with our matching system and genetic counseling, constitutes a powerful tool to avoid more than 600 mendelian diseases in the offspring of patients undergoing ART. (Fertil Steril® 2015;104:1286–93. ©2015 by American Society for Reproductive Medicine.)

Key Words: Assisted reproductive technology, carrier, genetic screening, infertility, next-generation sequencing

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In 2010, 48 million couples were affected by infertility; this incidence is expected to increase as parenthood is postponed (1). In many cases infertility can be surmounted by the use of assisted reproductive technologies (ART), which has enabled the birth of more than 7 million children worldwide (2); indeed, 2%–6% of total births in European countries are achieved through ART (3). The estimated number of ART cycles per year worldwide is approximately 1.5 million. Efforts and research in this area focus on achieving not only a pregnancy, but a genetically healthy baby at home—which is becoming the only metric for success in the field. In this sense, preconception health counseling and diagnosis is increasingly recognized as a critical medical component to prevent diseases in the offspring to reduce suffering, health care disparities, and therapeutic extra cost in the future generation.

Population-based carrier screening for single-gene disorders has been proposed since the 1960s. In the last two decades close to 1,150 recessive genes that cause mendelian diseases have been identified (www.ncbi.nlm.nih.gov/omim). Although rare individually, in developed countries these diseases collectively account for 20% of infant mortality and approximately 10% of pediatric hospitalizations (4, 5). Initially, prospective parents from high-risk populations were counseled and offered gene-by-gene carrier screening methods to search for frequent mutations. This strategy resulted in remarkable declines in the incidence of severe diseases common in those populations. Now, the advent of high-throughput next-generation sequencing (NGS) makes a comprehensive preconception screening panel more feasible, allowing for the possibility of affordable testing for a wide range of conditions that a family history will never detect. Next-generation sequencing technologies are powerful tools and methods for capturing targeted or arbitrary subsets of a genome. Each researcher or clinician can determine the appropriate high-throughput sequencing approach to sequence DNA or RNA and analyze sequence variation. For instance, the entire genome or the entire exome (protein-coding regions) can be sequenced or can be customized to sequence targeted regions or genes of interest. In this study we used NGS for targeted DNA sequencing and subsequent analysis of a set of genes causing mendelian disorders.

Preconception carrier screening allows couples to consider the most complete range of reproductive options. Knowledge of the risk of having an affected child may influence a couple's decision to conceive or to consider preimplantation genetic diagnosis (PGD), prenatal genetic testing, or the use of donor gametes to prevent disease in the child. A pioneer preconception carrier screen for 448 severe recessive childhood disease genes was developed using target enrichment and NGS (6). In that screen, the average carrier burden of severe recessive mutations was 2.8 per genome, ranging from 0 to 7 (mode of 2) (6). Another group has developed an integrated NGS workflow for specific ethnic groups that meets the main requirements for carrier screening; the protein-coding regions of 15 genes from genomic DNA isolated from whole blood are sequenced using the Illumina HiSeq 2000 (7). These pioneer developments demonstrate both the feasibility and importance of incorporating an expanded, NGS-based carrier screen into the clinic.

Here we report the development and clinical results of a preconception carrier genetic screening test (CGT) for severe recessive and X-linked childhood diseases, based on NGS target enrichment. This test has been clinically applied in couples undergoing ART with their own gametes, as well as in a large ovum and sperm donor program to avoid severe single-gene diseases of childhood. In addition, we report the creation of a blind matching program between donors and recipients that avoids both ethical issues and the unnecessary discarding of donors beyond those that carry an X-linked pathogenic variant.

MATERIALS AND METHODS

Study Design

Here we report the retrospective analysis of results obtained from CGT application in 2,570 individuals; the cohort comprised 1,170 gamete donors from the ovum and sperm donor programs; 1,124 individuals who were the partner of the patient receiving the donated gamete; and 276 individuals from 138 couples seeking ART using their own gametes. Permission to perform this study was obtained by the ethics committee (institutional review board) of Instituto Valenciano de Infertilidad (code 1411-VLC-075-CS).

Disease Selection

To establish a pan-ethnic NGS-based comprehensive carrier screening method, pathologic conditions were carefully chosen on the basis of clinical utility, disease incidence, and recommendations of professional societies for genetic screening. The gene panel covers a total of 549 genes implicated in

623 disease phenotypes in the OMIM (Online Mendelian Inheritance in Man) database, a comprehensive compendium of human genes and phenotypes freely available (www.ncbi.nlm.nih.gov/omim), which involves diseases in all human body systems (Supplemental Table 1, available online). The selected conditions were deemed to meet American College of Medical Genetics and Genomics (ACMG) criteria for implementation of genetic testing for rare disorders (8). In addition, literature and database searches and professional guidelines and recommendations (American College of Obstetrics and Gynecology Committee on Genetics) (9–11) were followed and reviewed to further select inherited conditions. We also included conditions with known molecular bases that were reported to be included in PGD (12) and prenatal diagnosis programs (9). Broadly, we included recessive and X-linked childhood diseases with severe and highly penetrant phenotypes that would most probably modify clinical counseling for preventive measures and family planning by prospective parents. We also selected some high-prevalence monogenic diseases with moderate phenotypes, disabilities that have a lifelong impact on the quality of life of the patient, such as severe hearing loss and blindness. Furthermore, to improve clinical utility we added a separate test for screening five high-prevalence diseases that cannot be currently identified by NGS (see "Complementary Molecular Tests" section).

Although we did not include adult-onset inherited cancer syndromes, severe childhood diseases caused by genes that may also have an impact on cancer development were included. The possibility for reporting potential incidental findings for breast cancer susceptibility gene *BRCA2* (included here owing to risk for biallelic mutations causing Fanconi anemia, a chromosome instability childhood syndrome) was discussed during genetic counseling both before and after carrier screening testing.

DNA Extraction

Genomic DNA samples were isolated from peripheral blood samples collected in ethylenediaminetetraacetic acid using the MagNA Pure Compact Nucleic Acid Isolation kit-large volume (Roche Diagnostics). Quantity and quality of DNA in each sample was determined by a Nanodrop1000 spectrophotometer and Qubit 2.0 Fluorometer (Thermo Scientific), according to the manufacturer's instructions.

Gene Panel Design, Targeted Gene Sequencing, and Data Analysis

Massively parallel sequencing was performed on the Hiseq2000 platform (Illumina). Briefly, sequence-enrichment DNA probes were designed and commercially obtained using the NimbleDesign system (Roche NimbleGen) and included all coding exons with flanking 30-bp intronic sequences of the targeted gene set of 548 genes. The targets comprised 8,871 exons with 1.91 Mb, including flanking intronic sequences; probe design directly covered 98.85%, with a principal coverage of 99.9% regions of interest. Targeted exon captures were performed according to a protocol described previously (13). Each DNA sample was indexed during the library preparation, and each 20–25 samples were sequenced (PE100) on one lane of the Hiseq2000 platform.

Sequence data analysis was performed using the bioinformatic pipeline, as follows. Briefly, the Illumina analysis pipepline (CASAVA1.8) was used for base-calling, in-house scripts were used to remove low-quality data and separate each barcoded data set, Burrows-Wheeler Aligner was used to map reads to the reference genome hg19, Genome Analysis Tollkit (Broad Institute) was used to detect single-nucleotide variants (SNVs) and small indels, an in-house pipeline was used to identify the deletion or duplication of exons in genes, and in-house scripts were used for annotation of variants.

Variant Interpretation

We used allele frequency to classify all detected variants as common or rare. Variants with an allele frequency $\geq 5\%$ in dbSNP (www.ncbi.nlm.nih.gov/SNP/), in the 1000 Genomes project (www.1000genomes.org/), or in BGI in-house control exomes were defined as common variants. Moreover, all identified DNA variants were used to create a proprietary CGT database to define our own set of common DNA variants following the same allele frequency criteria. Variants with a frequency $<5\%$ were considered rare variants. Common variants were categorized as polymorphisms.

For rare variants, the ACMG Standards and Guidelines (14) were followed to categorize sequence variations detected in the NGS panel. We also used a comprehensive catalog of high-penetrance variants underlying mendelian disorders (The Human Gene Mutation Database or HGMD) (15); The HGMD data were obtained from HGMD Professional release 2014.4. Potential functional rare variants between 1% and 5% that had an allele frequency higher than the estimated prevalence of the diseases in general populations, or that were detected in presumably healthy controls from the 1000 Genomes project, or detected in BGI in-house exomes as homozygous were categorized as likely polymorphisms. Rare missense SNVs and in-frame coding indels with an allele frequency lower than the estimated prevalence of the corresponding conditions with no homozygous status ever detected in controls, but not reported in patients or reported but without clear evidence of causing disease, were classified as variants of unknown significance (VOUS). Finally, rare variants—normally below 1%—with severe functional impact (frameshift deletions, nonsense SNVs, and splice site variants) with allele frequency below the corresponding disease prevalence with homozygous status ever detected in controls were classified as likely pathogenic. Mutations reported in one or more patient(s) with confident medical evidence were classified as pathogenic. Furthermore, the CGT database was used to curate potentially unreliable entries (6); rare variants defined as disease variants in the HGMD were classified as VOUS if the allele frequency ($1 <$ minor allele frequency ≤ 5) was higher than the estimated prevalence.

Complementary Molecular Tests

To meet our main purpose of clinical utility, we applied complementary gene testing covering some

high-prevalence mutations, technically unidentified by the NGS method. We tested for spinal muscular atrophy (*SMN1* mutation: exon 7 deletion), α thalassemia (mutations: –SEA, -α3.7, -α4.2, –FIL, and –THAI), and congenital adrenal hyperplasia due to 21-hydroxylase deficiency (*CYP21A2* mutations: c.[710T>A;713T>A;719T>A], c.844G>T, c.955C>T, c.1069C>T,c.1360C>T, c.92C>T; c.293-13A/C>G (c.655A/C>G), c.518T>A, and c.923dupT). In DNA samples from women, we also tested for Fragile X (*FMR1* CGG-expansion) and Hemophilia A (F8 intron 1 and 22 inversions). Different methods, described elsewhere, were used for these independent analyses (Table 1) (16–21).

Protocol for Gamete Donor Selection before CGT

After appropriate genetic counseling and to implement an efficient and cost-effective system for CGT, the following protocol was applied in the gamete donation program. First, donors were microbiologically screened according to the Spanish Reproductive Law (14/2006). Then, cytogenetic karyotyping and Fragile X testing (females only) was performed to detect carriers of potential reproductive risk; donors with an abnormal karyotype and women carrying a premutated *FMR1* allele were excluded from the donation program. Finally, CGT was performed.

RESULTS

Evaluation of Quality Metrics

To validate the performance of our customized CGT panel, we evaluated key quality metrics considered for NGS-based clinical testing (22). First, using the YH genome DNA sample (first individual sequenced in the Yan Huang [YH] Chinese genome project) as reference material, we evaluated the sequencing depth required for detecting variants at high sensitivity and accuracy in the targeted exons. We found that a sequencing depth >200-fold could provide a genotyping sensitivity for >98% SNVs on targeted sequences (>20 depth) with an accuracy rate >99.95%. Second, we assessed the intra- and inter-run reproducibility of the NGS panel performance by replicate experiments using the same YH genome sample. High consistency in coverage rate, genotype sensitivity, and SNV detection accuracy were observed for both intra- and inter-run replicate experiments (Supplemental Tables 2 and 3). Third, to evaluate the power of mutation detection, we simulated 100 genomes harboring a set of 3,283 pathogenic mutations (Supplemental Table 4) that were collected from database and literature evidence for the 548 genes as described in Materials and Methods. The sensitivity, specificity, and accuracy of mutation detection were >99% at a simulated sequencing mean depth of 200-fold using the described NGS panel (Supplemental Table 5).

Preclinical Validation of the CGT

To further evaluate the power of mutation detection in the clinical context, a set of DNA samples previously characterized by Sanger sequencing was included as reference samples for clinical validation. They consisted of a mix of de-identified DNA samples from our single-gene disorders PGD program that were positive for mutations affecting 27 genes of interest, as indicated in Table 2. Briefly, we sequenced DNA samples from 48 patients carrying known mutations using the NGS panel in a blind test manner. Mutations were correctly detected in 47 of 48 samples (Table 2). The undetected mutation (c.1695del11; *IDUA* gene) was located within an uncovered region in the NGS panel sequencing. Overall, the analytical sensitivity was >99%, with an estimated clinical sensitivity of 98%.

CGT Results and Clinical Outcomes for Couples Undergoing ART

We performed a total of 2,570 CGTs in patients and gamete donors undergoing carrier testing in fertility clinics. As estimated for validations, the average sequencing depth for clinical samples was >200-fold, covering 98% of the targeted sequences with 20 or more reads (Table 3). The analyses comprised 1,170 gamete donors (926 females [79%] and 244 males [21%]), corresponding to 45.5% of the tests performed. Another 1,400 patients (54.5%) were screened, of whom 1,124 (80.3%) were the partners of the patients receiving gametes and 276 (19.7%) were from 138 couples

TABLE 1

Complementary molecular tests included in the CGT.

Disease	Genes	Mutations	Method	References
Alpha thalassemia	*HBA1* and *HBA2*	–SEA, -α3.7,-α4.2,–FIL and –THAI	GapPCR analysis	15, 16
Congenital adrenal hyperplasia due to 21-hydroxylase deficiency	*CYP21A2*	c.[710T>A;713T>A;719T>A], c.844G>T, c.955C>T, c.1069C>T,c.1360C>T, c.92C>T; c.293-13A/C>G (c.655A/C>G), c.518T>A and c.923dupT	PCR and Sanger sequencing	17
Hemophilia A	*F8*	Inversion intron 1; inversion intron 22	Long-PCR analysis	18, 19
Spinal muscular atrophy	*SMN1*	Deletion of exon 7	qPCR	20
Fragile X syndrome	*FMR1*	TNR (CGG) expansión	PCR and fragment analysis	

Note: PCR = polymerase chain reaction; qPCR = quantitative PCR.

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TABLE 2

Mutation detection for 48 validation samples.

IGx ID	Gene	Inheritance	Mutation (Sanger sequencing)	NGS test result		
				Mutation	Depth of mutation allele	Ratio of mutation allele depth
113.1	*OTC*	X	c.674C>T	c.674C>T	99	0.57
356.1	*PCCA*	AR	p.G631R	G631R	129	0.52
356.2	*PCCA*	AR	p.G631R	G631R	118	0.54
395.1	*HBB*	AR	cd39	cd39	122	0.49
395.2	*HBB*	AR	IVS1-6	IVS1-6	109	0.44
435.1	*HBB*	AR	IVS1-NT110	IVS1-NT110	127	0.51
460.2	*CFTR*	AR	D1445N	D1445N	107	0.46
473.2	*CFTR*	AR	N1303K	N1303K	65	0.43
502.1	*CFTR*	AR	M952T	M952T	124	0.5
502.2	*CFTR*	AR	R117H	R117H	120	0.48
1220.1	*SLC26A2*	AR	c.835C>T	c.835C>T	98	0.44
1200.1	*CFTR*	AR	D579G	D579G	98	0.52
1191.2	*ABCA12*	AR	c.6639_6642del	c.6639_6642del	92	0.49
1187.1	*GAA*	AR	IVS1-13T>G	IVS1-13T>G	105	0.5
1187.2	*GAA*	AR	c.1115A>T	c.1115A>T	115	0.5
1172.1	*MY07A*	AR	c.4543_4551delGAGATCATGinsCA	c.4543_4551delGAGATCATGinsCA	95	0.43
1172.3	*MY07A*	AR	c.4544_4551delins CA & c.6024delG	c.4544_4551delins CA & c.6024delG	107 & 118	0.44 & 0.48
1162.1	*AHI1*	AR	c.2168G>A	c.2168G>A	127	0.51
1152.1	*GJC2*	AR	p.His252Asp	p.His252Asp	113	0.47
1139.1	*ABCD1*	X	c.1546delTGTTCC	c.1546delTGTTCC	91	0.45
1126.1	*MUT*	AR	c.655A>T	c.655A>T	99	0.41
1116.2	*ERCC6*	AR	c.2612T>C	c.2612T>C	118	0.47
1096.2	*LAMC2*	AR	c.2074C>T	c.2074C>T	107	0.51
1063.2	*CFTR*	AR	G542X	G542X	128	0.54
1055.1	*MMACHC*	AR	c.271dupA	c.271dupA	118	0.57
942.2	*PEX1*	AR	c.1145delG	c.1145delG	119	0.48
916.1	*RPGR*	X	c.1579_1581del & c.1598C>T_CIS	c.1579_1581del & c.1598C>T_CIS	79 & 92	0.43 & 0.43
916.3	*RPGR*	X	c.1579_1581del & c.1598C>T_CIS	c.1579_1581del & c.1598C>T_CIS	118 & 127	0.99 & 0.99
872.2	*CFTR*	AR	2789+5G>A	c.2789+5G>A	104	0.49
870.1	*FANCA*	AR	c.3239G>A	c.3239G>A	134	0.54
870.2	*FANCA*	AR	c.233_236del	c.233_236del	124	0.51
817.1	*PKLR*	AR	c.1481T>C	c.1481T>C	47	0.6
817.2	*PKLR*	AR	c.1675C>T	c.1675C>T	106	0.44
743.1	*ABCD1*	X	c.1801-1802delAG	c.1801-1802delAG	101	0.48
737.1	*GJB2*	AR	35delG	35delG	87	0.46
737.2	*GJB2*	AR	W77R	W77R	130	0.52
737.3	*GJB2*	AR	35delG&W77R	35delG&W77R	126 & 129	0.52 & 0.52
718.1	*CFTR*	AR	G542X	G542X	125	0.5
694.1	*RS1*	X	c.599G>A	c.599G>A	110	0.44
678.2	*FANCA*	AR	c.4130C>G	c.4130C>G	118	0.47
671.1	*IDUA*	AR	1695del11	no change		
664.1	*MECP2*	X	R270X	R270X	132	0.53
619.1	*NPC1*	AR	IVS23+5G>A	IVS23+5G>A	134	0.54
582.2	*IGHMBP2*	AR	C496X o R788X	C496X o R788X	124	0.5
568.1	*KCNJ1*	AR	Cys49Arg	Cys49Arg	126	0.51
534.1	*NPC1*	AR	R1059X	R1059X	107	0.43
1140.1	*CFTR*	AR	dF508	dF508	110	0.45
1140.2	*CFTR*	AR	dF508	dF508	107	0.43

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using their own gametes. In total we detected 1,796 unique pathogenic or likely pathogenic variants; 13,785 variants of unknown clinical significance (VOUS) were defined. From the 2,570 patients investigated, 2,161 (84%) were positive for at least one pathogenic variant. The average carrier burden of recessive or X-linked conditions was 2.3 mutations per sample.

For the 138 couples undergoing ART using their own gametes, our CGT identified seven cases with known pathogenic or likely pathogenic variants in both members, representing 5% of the couples investigated (Table 4). These couples received genetic counseling, and PGD was recommended. Specifically, 6 female patients out of 287 were positive for X-linked disorders, corresponding to approximately

TABLE 3

Carrier screening for 2,570 samples.

Variable	Mean	SD
Sequencing depth	187.51	33.98
Coverage of targeted sequences (%)	99.13	0.03
$\geq$1× (%)	99.13	0.03
$\geq$20× (%)	97.76	0.03
Variants detected	1038.96	62.65
SNV	1003.72	61.98
Indel	35.23	5.49
Carrier burden	2.28	
NGS_pathogenic	2.06	
Complementary molecular test	0.22	

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2% of this cohort. Preimplantation genetic diagnosis was suggested and accepted for their clinical management.

Carrier Burden and Blind CGT Match in the Gamete Donation Program

Gamete donors who tested microbiologically negative were subjected simultaneously to karyotype analysis and Fragile X (females only) investigation, resulting in an abnormal karyotype in 6% of them, who were then rejected as donors. The rest underwent CGT, starting with Fragile X. Eighteen female donors were additionally excluded from the program because they carried a pathogenic or likely pathogenic variant in an X chromosome gene, representing 1.94% of the total tests requested. They received information on the adverse finding, including genetic counseling and discouragement from entering the donation program.

The remaining donors (1,162 of 1,170 initially screened) were included in a blind-matching, informatically controlled database. By request, the match system always displayed a set of donors genetically compatible with the patient requesting gamete donation.

DISCUSSION

We have described the validation and subsequent clinical use of a comprehensive carrier genetic screening test and its clinical translation to infertile couples wishing to conceive through ART. This test is built upon the many advantages offered by next-generation DNA sequencing platforms. Next-generation sequencing platforms are widely available and, relative to Sanger sequencing, enable lower-cost sequencing at a faster rate for accumulating massive sequence reads. Coupled with improved computational and bioinformatics data analysis, this sequencing technology has entered the clinic. Here, we sequenced hundreds of genes simultaneously, using a proprietary genetic assay that in an independent validation performed with samples containing all classes of known mutations indicated that the test had an analytical sensitivity >99% and an estimated clinical sensitivity of 98%.

To be clinically useful, sequence analysis must reliably distinguish disease-causing genetic variants from background (nonpathogenic variants) present in all human genomes. Here, we detected 4,925 deleterious variants (1,796 unique) and 35,537 VOUS. Using this information, 2% of female donors were rejected from the program because of being carriers for an X-linked mutation. The identification of a deleterious mutation in autosomal recessive (AR) genes, however, did not require the rejection of additional donors because we applied a blinded matching system. Following the recommendations of a recent task force publication that identified ethical issues in genetic screening of gamete donors (23), we designed a blinded matching system that allows us to prevent diseases in the offspring, while avoiding unethical donor rejections. This is especially important because the use of our CGT identified that only 15% of the samples tested negative for all pathogenic or likely pathogenic variants. Moreover, in addition to finding donors at risk of transmitting variants for rare AR disorders, the designed panel enabled risk profiling for certain low-penetrance mutations. During genetic counseling, information for these types of mutations was also given; for some of these variants (i.e., a donor with p.H63D found in the HFE gene; hemochromatosis) we did not consider recommending future preventive measures, like testing partners or PGD in the future. However, in gamete donation cycles we still included them for matching in case of necessity (i.e., to avoid a donor with the previous variant for a recipient carrying p.C282Y in the HFE gene).

All donors received the corresponding genetic report and those free of pathogenic variants or carrying only AR mutations were accepted for the gamete program; when mutations were detected, a proper genetic counseling was provided, with

TABLE 4

Diseases and mutations for couples tested positive (high risk) after CGT carrier screening.

Disease	Inheritance	Gene	Variant_male cHGVS	Variant_male pHGVS	Variant_female cHGVS	Variant_female pHGVS
Hemophilia A	XL	*F8*			Intron22 inversion	
Smith-Lemli-Opitz syndrome	AR	*DHCR7*	c.292C>T	p.Gln98X	c.292C>T	p.Gln98X
Retinitis pigmentosa 19	AR	*ABCA4*	c.5882G>A	p.Gly1961Glu	c.5908C>T	p.Leu1970Phe
Autosomal recessive polycystic kidney disease	AR	*PKHD1*	c.4165C>A	p.Pro1389Thr	c.3407A>G	p.Tyr1136Cys
Fragile X syndrome	XL	*FMR1*			62 CGG repeats	
Autosomal recessive polycystic kidney disease	AR	*PKHD1*	c.3407A>G; c.9866G>T	p.Tyr1136Cys; p.Ser3289Ile	c.10036T>C	p.Cys3346Arg
Argininosuccinic aciduria	AR	*ASL*	c.539T>G	Leu180Arg	c.539T>G	Leu180Arg

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emphasis regarding risk for their own family planning. Recommendations for testing their partner before pregnancy were also clearly manifested.

For the purpose of clinical testing and prevention, we have also included the results for 138 couples seeking assisted reproduction. Of these, 5% were at high risk because partners shared recessive mutations in the same gene. All seven couples were offered preventive programs, including recommendation for embryo PGD. Indeed, four couples are currently performing PGD. According to our initial focus and design, types of infertility and clinical take-home baby rates were not affected or influenced by this carrier genetic screening test. As previously indicated, information and counseling were provided to all participants, but was not necessary for other members of the family because this analysis was focused on preventing diseases of the offspring. Still, recommendations for testing other family members were clearly indicated for all positive cases.

On the basis of the previous figures, we can estimate that approximately 56 additional high-risk treatments (5% of 1,124) (i.e., ART potentially with high genetic risk to transmit mutation/s to the offspring) have been prevented in our program by using the CGT combined with the matching system. Altogether, the implementation of this CGT for couples and patients requiring gamete donation may have potentially prevented 1.25% affected babies born after ART. Importantly, this number considers only the pathogenic variants. Although the majority of VOUS are eventually discovered to be non–disease-causing, some are pathogenic. However, VOUS present a challenge to the clinician in how to appropriately guide the medical care of a patient in the context of an inconclusive test result. As a general rule, VOUSs were not reported to individuals. Exception was made for those couples in which the partner of the VOUS carrier also had a pathogenic variant in the same gene. Counseling included intensive explanation regarding actual knowledge for VOUS interpretation; the option to ask for PGD was introduced sometimes, and in that case the genetic counselor always cleared up doubts about missing information regarding impact on phenotype. Nevertheless, we still worked to further reclassify these VOUS, which requires a costly labor- and data-intensive effort using various lines of evidence; in addition, we used, conservatively, the advantage of having all donors tested and the blinded matching system to avoid combinations of VOUS-pathogenic pairs for a given gene when selecting a donor for a patient, thus improving the clinical utility of the test.

The application of a generalized genetic carrier screening is a powerful tool, especially for certain high-risk ethnic populations. Recent statements from professional organizations promote the use of expanded carrier screening in women of reproductive age before conception. Gamete donors should undergo carrier screening before their use as part of all screening programs (24). Indeed, recent works have also used NGS as the method of screening because it is not limited to a small number of mutations, thus providing the possibility of finding a much larger set of sequence variations across many ethnic groups (25, 26). However, the use of this powerful technology also create one of the most challenging areas in the carrier screening arena, the interpretation and reporting of the variants detected. Many algorithms of decision have been suggested, attempting to integrate automatized bioinformatics pipelines in the detection of the disease-causing variants among all the detected variants and in the definition of their pathogenicity. Most of these algorithms are based on steps like filtering by allele frequency in public and/or in-house databases, functional impact in the protein, and genotype–phenotype coherence. Although all these filters and parameters are not conclusive, they help to do a better interpretation and categorization of the detected variants. Indeed, the integration of the bioinformatics pipeline is mandatory in this type of genetic test, to automatize and standardize the analysis and interpretation process. The carrier frequencies in our patient cohort are generally in agreement with the available literature. However, classification is based on available collections of disease alleles that are invariably imperfect—not complete for very rare disease-alleles, but they also contain entries that have been erroneously included as disease variants, as indicated by recent work (6). There is an urgent need to improve disease-allele annotation; more comprehensively annotated databases of pathologic or disease-associated variants will be required (27). Therefore, giving a definitive estimate of the number of disease-causing variants per individual is imperfect at this stage. In addition, considerable uncertainty remains regarding which sequences of the human genome are truly protein coding: even today we have an increasingly large number of hypothetical proteins. Then, the capture probes—for recovering DNA or gene regions of interest—can only target exons that have been identified so far. This and other issues like different capture and/or sequencing efficiency for different templates add further limitations to give a definitive estimation of the carrier burden in humans. A negative result does not eliminate risk to offspring, even assuming a correct design and performance. These limitations must be included in the informed consent and clearly explained to both patients and clinicians ordering this type of test. Finally, the informed consent must include a pretest personal decision regarding any potential incidental finding.

In conclusion, we have developed a comprehensive carrier genetic screening test that, combined with our matching system and genetic counseling, constitutes a powerful tool to avoid more than 600 mendelian diseases in the offspring of patients undergoing ART. Altogether, the two main important take-home messages for the clinician are, first, without screening, approximately 2% of donors entering the egg donation programs are carrying X-linked mutations; and second, by using our expanded genetic screening we have prevented the birth of 1.25% of genetically affected babies without the need to reject a significant number of gamete donors.

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