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In vitro screening of embryos by whole-genome sequencing: now, in the future or never?

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STUDY QUESTION: What are the analytical and clinical validity and the clinical utility of *in vitro* screening of embryos by whole-genome sequencing?

SUMMARY ANSWER: At present there are still many limitations in terms of analytical and clinical validity and utility and many ethical questions remain.

WHAT IS KNOWN ALREADY: Whole-genome sequencing of IVF/ICSI embryos is technically possible. Many loss-of-function mutations exist in the general population without serious effects on the phenotype of the individual. Moreover, annotations of genes and the reference genome are still not 100% correct.

STUDY DESIGN, SIZE, DURATION: We used publicly available samples from the 1000 Genomes project and Complete Genomics, together with 42 samples from in-house research samples of parents from trios to investigate the presence of loss-of-function mutations in healthy individuals.

PARTICIPANTS/MATERIALS, SETTING, METHODS: In the samples, we looked for mutations in genes that are associated with a selection of severe Mendelian disorders with a known molecular basis. We looked for mutations predicted to be damaging by PolyPhen and SIFT and for mutations annotated as disease causing in Human Genome Mutation Database (HGMD).

MAIN RESULTS AND THE ROLE OF CHANCE: More than 40% of individuals who can be considered healthy have mutations that are predicted to be damaging in genes associated with severe Mendelian disorders or are annotated as disease causing.

LIMITATIONS, REASONS FOR CAUTION: The analysis relies on current knowledge and databases are continuously updated to reflect our increasing knowledge about the genome. In the process of our analysis several updates were already made.

WIDER IMPLICATIONS OF THE FINDINGS: At this moment it is not advisable to use whole-genome sequencing as a tool to set up health profiles to select embryos for transfer. We also raise some ethical questions that have to be addressed before this technology can be used for embryo selection.

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† The authors consider that the first two authors should be regarded as joint First Authors.

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Introduction

Genetic testing of preimplantation embryos is a generally accepted approach in the context of preimplantation genetic diagnosis (PGD): patients with a known risk to transmit a specific genetic condition, or with a known chromosomal rearrangement, can opt for PGD to select embryos without the relevant disease-causing mutation. A second application of genetic testing of embryos is preimplantation genetic screening (PGS) for aneuploidy. Although not yet sufficiently proven by randomized controlled trials, this is offered by some centers to subfertile patients undergoing IVF as a treatment of infertility or as part of PGD, with the aim of improving their chances of a successful pregnancy. Whole-genome sequencing (WGS) and analysis, which determines and analyzes the entire DNA sequence of an individual in one procedure, has been performed in single cells and single blastomeres (Navin *et al.*, 2011; Xu *et al.*, 2012; Voet *et al.*, 2013). WGS (followed by a targeted analysis) could be a generic approach for PGD, avoiding time-consuming and labor-intensive customized PGD workups. WGS might also be an elegant alternative method for PGS, since full chromosomal aneuploidies and expected segmental imbalances associated with chromosome rearrangements can be easily identified (Harper and Sengupta, 2012). As WGS costs are now approaching the costs for array-based single-cell PGD or PGS, WGS may become a useful auxiliary technique for embryo testing as already performed in such context (Baslan *et al.*, 2012; Hens *et al.*, 2013; Simpson *et al.*, 2013).

In addition to these applications, WGS could theoretically be used to extend considerably the scope of embryo testing. This would entail a widening of the aims of the procedure. In addition to helping people either to have a child without a specific disorder (as in PGD), or to attempt to increase the chances of a successful IVF pregnancy (as in PGS for aneuploidy), the aim of WGS in embryo testing would be to ensure that children born after IVF or IVF/PGD are free from major disorders. Unlike classical PGD, embryo testing with these aims would be a form of medical screening, as it is a form of indiscriminate genetic testing without clinical data. One of the accepted criteria for responsible screening is that there should be a suitable test (Wilson and Jungner, 1968). This means that both the *analytical and clinical validity* of the test must have been demonstrated. The analytical validity of a genetic test is its ability to determine accurately the genotype of interest. Clinical validity is the accuracy with which the test can then predict a phenotype. If the test performs poorly in these regards, this will adversely affect the *clinical utility* of the screening. This last concept refers to the balance of aim-related advantages and unavoidable disadvantages (drawbacks and costs), and is as such directly linked with the ethical acceptability of screening programs (Sanderson *et al.*, 2005; Dondorp *et al.*, 2010). In this paper, we assess the analytical and clinical validity of WGS-based testing as a necessary (though not a sufficient) condition for the clinical utility and ethical acceptability of extended or comprehensive embryo screening using this technology.

Materials and Methods

We ascertained whether state-of-the-art technology and know-how could adequately distinguish benign polymorphisms from disease-causing mutations and help in deciding which embryo to transfer. We investigated how many apparently healthy adults carry mutations predicted to be damaging or annotated as disease causing—under the rationale that if such mutations were sufficient to cause the severe early-onset phenotype (and therefore predictive in a screening setting), they should be absent from the exome of apparently healthy adults.

Disease selection

We obtained the Online Mendelian Inheritance in Man (OMIM; McKusick-Nathans Institute of Genetic Medicine, 2012) list of diseases with a known molecular basis, consisting of >3000 diseases, from the OMIM website. This list was cross-referenced with the Human Phenotype Ontology database (Robinson and Mundlos, 2010) to provide an overview of diseases and their associated phenotypes. The resulting set contained 2172 diseases from which we selected diseases characterized by dysmorphology and early-onset symptoms. By selecting early-onset disorders we ensure that individuals affected by one of these disorders would already show symptoms at the time of sequencing. In addition, only diseases that had the inheritance annotated in OMIM as 'autosomal dominant', 'autosomal dominant type; high penetrance' and 'autosomal recessive' were selected. As a result 132 autosomal dominant and 215 autosomal recessive diseases were retained. The complete list of these diseases can be found in [Supplementary data, Tables SI and SII](#).

Samples

For our analysis, we used both private and publicly available samples from adult individuals who were considered healthy at the time of sequencing (i.e. who did not exhibit clear signs of a congenital disorder at the time of sampling). We had access to 42 exome sequences from in-house research samples. These are trio samples where the parents are considered healthy, as they are symptom free. The sequences of affected children were also available but were not used in the initial analysis. In addition to our high-quality exome sequences, we downloaded two freely available sets from the 1000 Genomes project (1000 Genomes Project Consortium, 2012) (1kG) and Complete Genomics (Drmanac *et al.*, 2010) (CG). The 1kG data came from the phase I integrated release version 3 from 30 April 2012, containing genotype calls for >1000 individuals. The data from CG were from 69 individuals, the sample names of which can be found in [Supplementary data, Table SIII](#). All these sequences are from people who do not express symptoms of the selected disorders and therefore considered 'healthy'. Because some of the samples of these publicly available sets are from trios, we removed the samples from related individuals from the data sets leaving 1004 samples from the 1kG data and 50 from the CG data.

Transcripts

To localize the mutations in the genes and the different transcripts of those genes, we used the Ruby Ensembl API (Strozzi and Aerts, 2011) to connect to the Ensembl core database version 70. In addition, we also

looked at transcripts present in the Consensus CDS Project (CCDS) database Hs37.3 (Pruitt et al., 2009).

Mutations predicted to be damaging

When analyzing genomes in the search of causative mutations, prediction algorithms are often used to predict the effect of a mutation on the protein. We used an in-house database called Annotate-it (Sifrim et al., 2012) that holds detailed information on the selected genes, to retrieve prediction scores from SIFT (Ng, 2003) and PolyPhen (Adzhubei et al., 2010) for the identified mutations. More information about these algorithms can be found in the [Supplementary data](#). Because PolyPhen and SIFT only give scores to missense mutations, we considered nonsense and splice site mutations also to be damaging in the analysis. Indels were not included in the analysis.

Mutations described to be damaging in literature

In addition to the prediction algorithms, we also looked for mutations that are described as disease causing in the literature. For information on these mutations, we used the Human Gene Mutation Database (HGMD) containing >70 000 disease-causing mutations (Stenson et al., 2003). For our analysis, we only selected those mutations with associations not considered tenuous by the curators of the HGMD and in the disease-causing category 'DM'.

Results

The results from this analysis show that many healthy individuals have mutations predicted to be damaging or annotated as disease causing in HGMD in genes associated with severe developmental disorders. For the 1kG samples all mutations are included, i.e. mutations that were found in both the low coverage and exome sequences. For the in-house data sets we only retained mutations with a read depth of at least 30× and phred score of 30. Relaxing these constraints leads to a higher number of mutations, which are described in [Supplementary data, Tables SIV and SV](#).

Autosomal dominant disorders: mutations predicted to be damaging

When looking for mutations predicted to be damaging, we found that depending on the data set, 98–100% of healthy individuals had damaging mutations in genes associated with the selected autosomal dominant disorders. In the 1kG data set, we found a median of 8 mutations (min. 3, max. 14), in the Complete Genomics data set a median of 9 mutations (min. 4, max. 14) and in the in-house data set a median of 2 mutations (min. 0, max. 5) per individual. In only one sample from the in-house data set no damaging mutations were found. The distribution of the number of mutations can be seen in Fig. 1. Because variants that are frequently found in the population are unlikely to cause these severe Mendelian disorders, we only retained the variants with a minor allele frequency (MAF) in the 1000 genomes of <1%. Applying this filter leads to a large decrease in identified variants but still 40–94% of individuals were found to carry damaging mutations. In the 1kG data set we found a median of 0 (min. 0, max. 5) mutations, in the Complete Genomics data set a median of 2 mutations (min. 0, max. 6) and in the in-house data set a median of 0 mutation (min. 0, max. 4) per individuals. The distribution of the number of mutations can be seen in Fig. 2.

As explained by MacArthur et al. (2012) faulty gene annotation is a likely cause for these genes containing a large number of deleterious mutations. An example of this can be found in EDARADD (Ectodysplasin-A receptor-associated death domain), which is the gene with the highest number of unfiltered damaging mutations in all data sets. Mutations in EDARADD cause the autosomal dominant form of ectodermal dysplasia, characterized by sparse hair, missing or abnormal teeth and the inability to sweat (Cluzeau et al., 2011). For this gene we found mutations in 83–98% of the samples. These mutations however are not annotated as disease causing in HGMD. In the 1kG data the most occurring mutation in EDARADD is at chromosome 1, position 236557771 G>A (dbSNP id: rs966365). So even though it is predicted to be damaging by both SIFT and PolyPhen, it is frequently found in that population (91%) suggesting an annotation error in the reference genome. Another observation we made was that most of the genes with the most common mutations have multiple transcripts and most have at least one transcript that is not affected by these mutations.

After filtering out the variants with a MAF in the 1000 genomes of >1%, we find differences in the percentage of samples showing mutations between the data sets. For instance, the most occurring variant in the in-house data set was found in NOTCH2, which is associated with Hajdu–Cheney syndrome, a disease characterized by coarse face, short neck, hirsutism, joint laxity and bone dysplasias (Ramos et al., 1998). In this case, mutations were found in 14% of the in-house samples, in 1% of the 1kG samples and 6% of the CG samples. The fact that a predicted damaging variant is frequently found in the local population but not in the 1000 genomes data set and dbSNP can indicate that the variant is a benign polymorphism in this population. An example of this is a variant that we identified at chromosome 3, position 98300354 (A>C) in CPOX, which is associated with hereditary coproporphria. This mutation is found in almost 10% of the in-house samples but does not occur in the other data sets and is not found in dbSNP.

In total we identified 323, 120 and 21 distinct mutations with a MAF of <1% in the 1kG data in 69, 59 and 18 genes in, respectively, the 1kG, CG and in-house data sets. An overview of the genes with the largest number of predicted damaging mutations and some phenotypic information about the disorder is shown in [Supplementary data, Tables SVI and SVII](#).

Autosomal dominant disorders: mutations present in HGMD

Taking only the mutations into account that are annotated as disease causing in HGMD for the severe disorders from our list, we identified mutations in 20% of the 1kG samples, 22% of the Complete Genomics samples and 12% of the in-house samples. The number of affected samples with curated disease-causing variants is thus much lower compared with those identified by the prediction programs. When looking at the in-house samples, we found that the mutations were considered to be damaging by either PolyPhen or SIFT but not by both. A list of the diseases of which causative mutations were identified can be found in [Supplementary data, Table SVIII](#).

In the in-house data, we identified a total of six heterozygous mutations in five individuals. One of these mutations (CM023740) had a MAF >1% in the 1000 genomes indicating a possibly suspicious annotation or a variant with reduced penetrance. At the time of the initial analysis, this mutation was annotated as causing extrahepatic biliary atresia, a

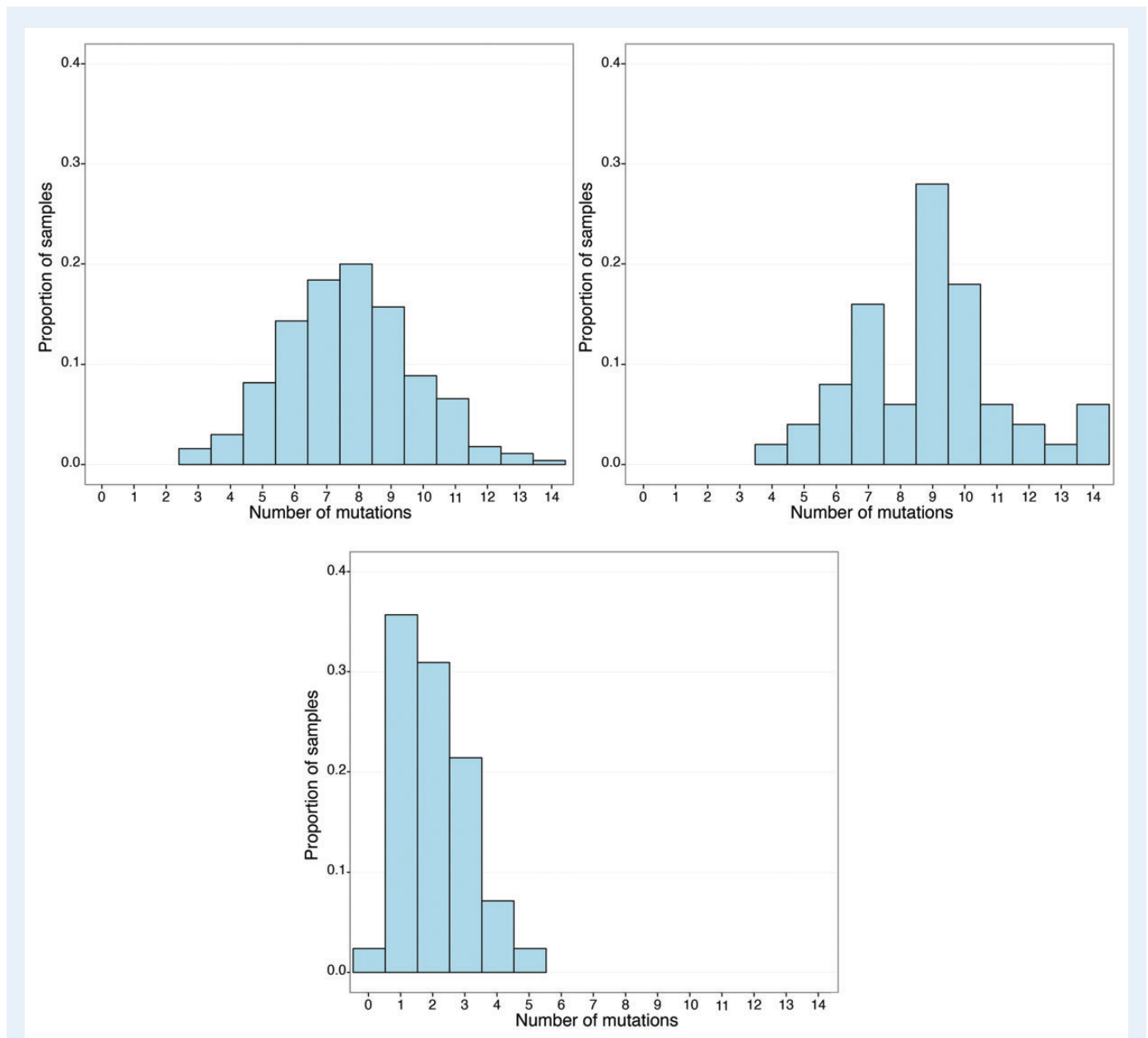


Figure 1 Histograms showing the proportion of samples with a certain amount of mutations that are predicted to be damaging. Samples are from the 1000 genomes, Complete Genomics and in-house (clockwise starting top left).

feature of Alagille syndrome, but it was removed from HGMD at a later time. For Greig cephalopolysyndactyly syndrome two distinct mutations were found in *GLI3*, i.e. P707S (CM970684) (Wild *et al.*, 1997) and I808M (CM990707) (Kalf-Suske *et al.*, 1999). In a functional analysis, both mutations were found to cause misregulation of *GLI3*-localization by Krauss *et al.* (2009). The mutation causing Rubinstein-Taybi syndrome—A981T (CM021081) in *CREBBP*—was identified by Coupry *et al.* (2002) in a set of 60 patients. Because these samples are part of trios, we also had access to the samples of the children. Out of a total of five children, four children were heterozygous for the same mutation as their parent(s) but also did not express the disease. The fact that these variants are present in apparently healthy individuals may hint towards (i) sequencing errors, (ii) false-positive entries in HGMD or (3) incomplete

penetrance of certain variants that would make them of low predictive value in a PGS context.

Autosomal recessive disorders: mutations predicted to be damaging

For autosomal recessive disorders, there are two categories of affected individuals. Either they are homozygous for a mutation or they are compound heterozygous. We found almost the same number of samples that were homozygous for damaging mutations in genes associated with autosomal recessive disorders as we found mutations for the autosomal dominant disorders. Approximately 98–100% of samples were homozygous for at least one mutation predicted to be damaging. The number of

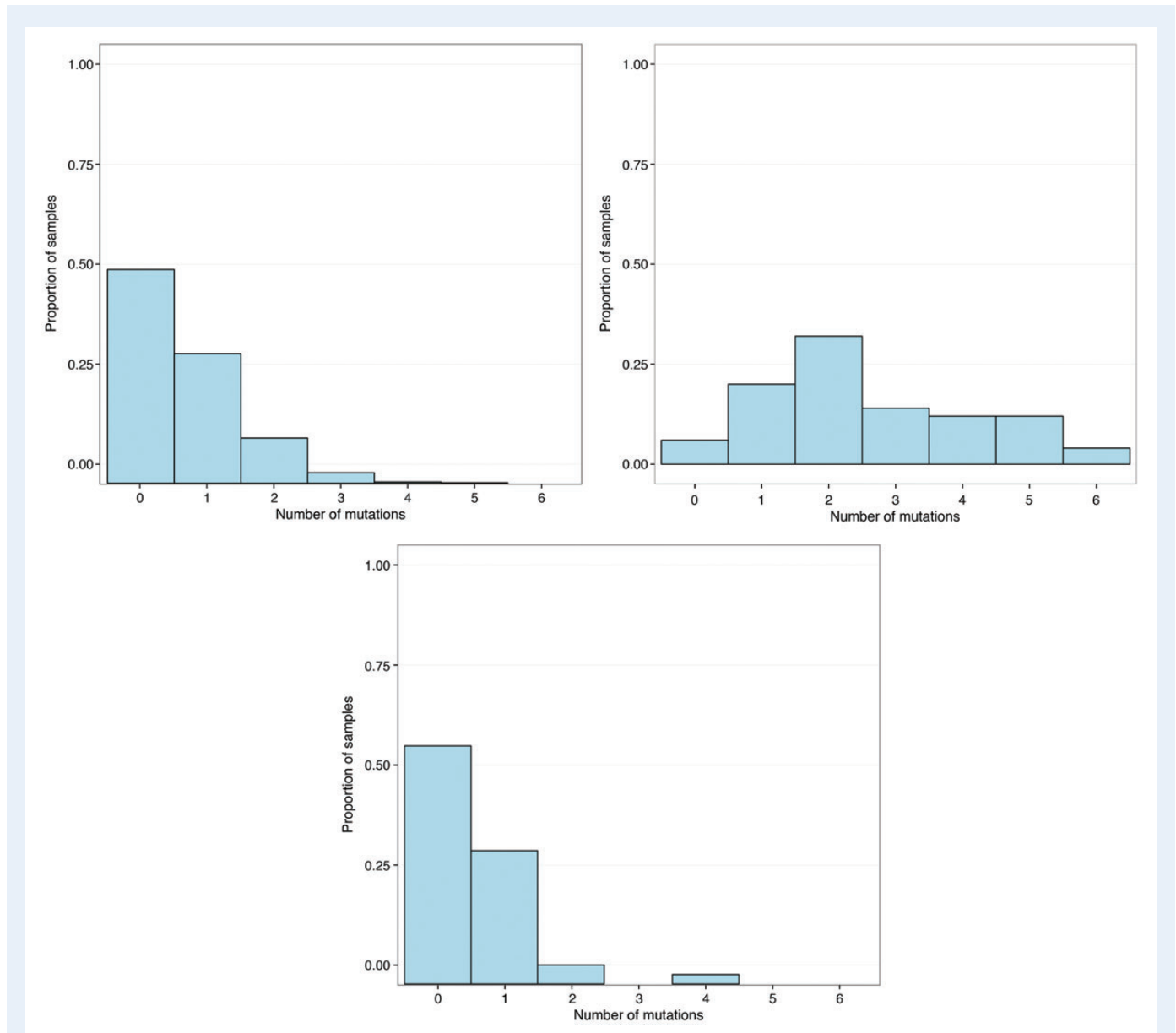


Figure 2 Histograms showing the proportion of samples with a certain amount of mutations that are predicted to be damaging and are found with a minor allele frequency of $<1\%$ in the 1000 genomes data. Samples are from the 1000 genomes, Complete Genomics and in-house (clockwise starting top left).

damaging mutations per individual was lower however. In the IkG data set we found a median of 4 mutations (min. 0, max. 9), in the Complete Genomics data set a median of 4 mutations (min. 1, max. 7) and also in the in-house data set a median of 4 mutations (min. 0, max. 7) per individual. The distribution of the mutation counts can be seen in Fig. 3. Limiting the variants to those with a MAF of $<1\%$ in the 1000 genomes data produced a large decrease especially in the IkG data set. Less than 2% of the samples are homozygous for a damaging mutation in the IkG data set and 56–69% in the CG and in-house data set. The median in the IkG data sets is 0 mutation (min. 0, max. 1), in the Complete Genomics data set 1 mutation (min. 0, max. 2) and in the in-house data set 1 mutation (min. 0, max. 1). The distribution of the mutation counts can be seen in Fig. 4. In total, we identified 17, 4 and 1 distinct homozygous mutations with MAF

in IkG of $<1\%$ in 16, 4 and 1 genes in, respectively, the IkG, CG and in-house data set. As for the autosomal dominant disorders, [Supplementary data, Tables SIX and SX](#) show an overview of the genes with the largest number of mutations and the corresponding diseases.

In addition to homozygosity, we also investigated the case of compound heterozygosity. Because no haplotype information was available for the in-house data set, we identified the number of samples that have two different mutations predicted to be damaging in each gene. In this case, the difference between the data sets is also large. While for some diseases a number of individuals are identified in one data set, there are no individuals with damaging mutations in another and vice versa. In this case, the gene NEB, which is associated with nemaline myopathy, showed the highest number of individuals that had at least two

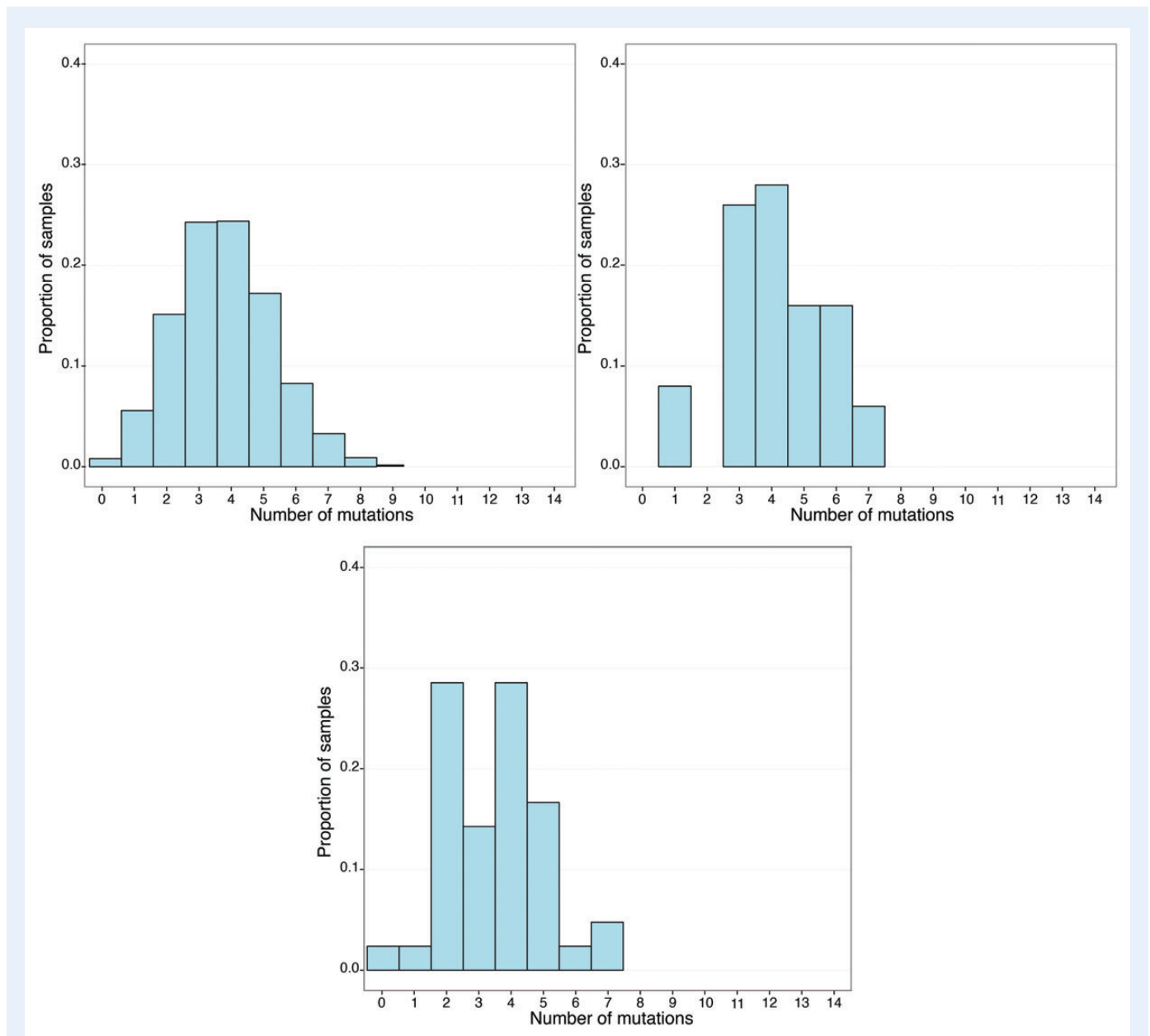


Figure 3 Histograms showing the proportion of samples with a certain amount of mutations that are predicted to be damaging. Samples are from the 1000 genomes, Complete Genomics and in-house (clockwise starting top left). Only homozygous mutations are counted.

distinct mutations with 2% of samples in the 1kG data set, 2% in the CG data set but no samples in the in-house data set affected. An overview of the number of affected samples and genes can be found in [Supplementary data, Table SXI](#).

Autosomal recessive disorders: mutations present in HGMD

For HGMD mutations, we found that the percentage of samples that are homozygous and therefore are expected to express the disease is much lower than with autosomal dominant diseases. In the in-house data set, no homozygous HGMD mutations were found. An overview of all affected genes can be found in [Supplementary data, Table SXII](#). The most

frequently identified mutation was found in 16 individuals in the 1kG data (1.8%) on chromosome 17, position 7915912 C>T (dbSNP id: rs34598902). This mutation was thought to be causative for Leber Congenital Amaurosis ([Zernant et al., 2005](#)), a disease characterized by congenital blindness that affects 10–20% of all blind children ([INSERM, 1997](#)). However, it was later found in the normal population ([Ito, 2004](#)) with a MAF in the 1000 genomes of 8.26%. While the mutation was annotated as disease causing in HGMD at the time of our analysis, it has been reclassified at a later time to the category 'DM?' indicating a tenuous association. Another mutation in the same gene at position 7912879 C>T (dbSNP id: rs28743021) was found in two individuals in the 1kG data who were homozygous for this mutation. This mutation is annotated as disease causing in HGMD based on a study by [Koenekoop et al. \(2002\)](#).

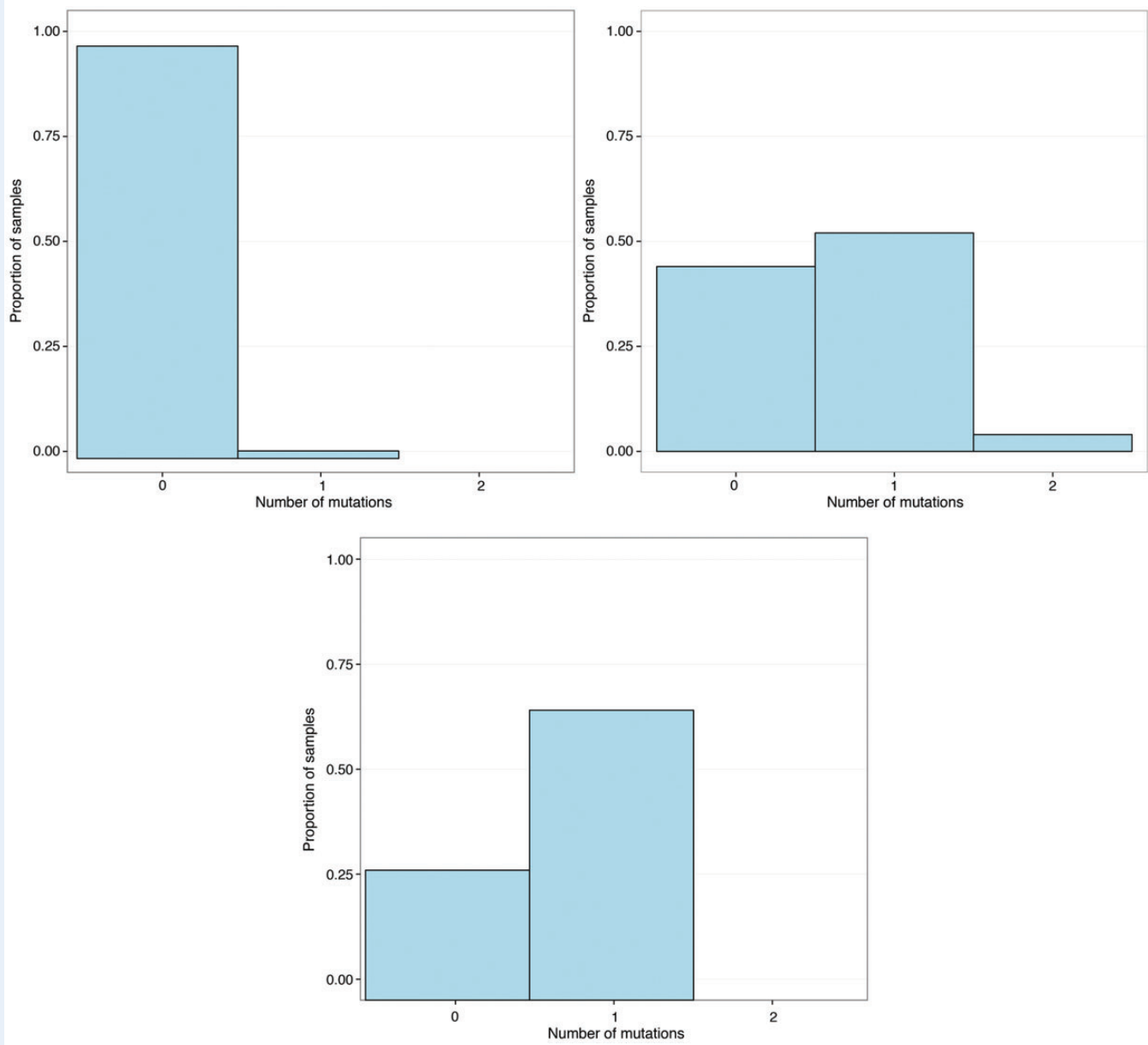


Figure 4 Histograms showing the proportion of samples with a certain amount of mutations that are predicted to be damaging and are found with a minor allele frequency of $< 1\%$ in the 1000 genomes data. Samples are from the 1000 genomes, Complete Genomics and in-house (clockwise starting top left). Only homozygous mutations are counted.

For compound heterozygous mutations, again counted as two distinct mutations, no positive samples were identified in the in-house data, only one in the CG data set with a MAF $> 1\%$ and a few in the 1kG data set. An overview of the number of affected samples is shown in [Supplementary data, Table SXIII](#).

Discussion

Analytical validity

The analytical validity of WGS-based embryo testing is constituted by the quality of single-cell sequencing and the accuracy of interpretation. At

present, single-cell genome-wide sequencing is not as good as sequencing based on multiple cells. On the assumption that this limitation will be overcome, we focus here on the accuracy of the interpretation of sequencing data.

We found that with increasing quality thresholds the number of individuals that carry mutations predicted to be damaging decreases in the samples from our in-house data set. Except for a common mutation found in the CG and in-house data set, we found fewer individuals with mutations predicted to be damaging in genes associated with autosomal recessive disorders. This may be because homozygous mutations occur less frequently than heterozygous mutations or it may be linked to the clinical validity of the test: autosomal dominant disorders are more

likely to show reduced penetrance or variable expressivity and therefore symptoms of the disorder might not be present in these individuals although the mutations are.

Clinical validity

More than 40% of genomes of healthy individuals in our study had a genetic mutation thought to be causative for severe autosomal dominant congenital disorders. Moreover, some healthy individuals were homozygous or compound heterozygous for mutations associated with autosomal recessive disorders. These results are in line with the results of Xue *et al.* (2012) and MacArthur and Tyler-Smith (2010) who studied loss-of-function and disease-causing variants in healthy individuals. Our analysis relies on current prediction programs and databases. It is obvious from this analysis that current programs predicting protein damage based on exonic sequence information alone show a number of false positives and will need to improve to enable proper future phenotype prediction (Sifrim *et al.*, 2013). In our results we see that filtering the identified mutations on the MAF in the 1000 genomes significantly reduces the number of identified mutations per individual. In the CG and in-house data set however, a larger percentage of individuals still show mutations. This suggests that these mutations might be local polymorphisms that are not identified in the 1000 genomes project. An in-depth knowledge of the variants found in the local population can therefore lead to improved filters. Finally, the HGMD database is frequently updated and thus the results of each analysis might be different with each version of the database.

A specific challenge for embryo screening is that predictions have to be made in the absence of phenotypic information. This is different from the use of WGS-based testing in the post-natal context where this technology is being introduced as a means of finding a diagnosis for existing patients with a phenotype that could not be clarified using more traditional diagnostic approaches. However, in embryo selection, genetic information is all that is available, except for classical PGD where the phenotype of the parents can be taken into account. We considered to what extent adding WGS-based preconception testing of the prospective parents might help fill in this lacuna by providing additional context information. This approach may perhaps be helpful where findings related to dominant disorders are concerned, as information about genotype and phenotype of the parents will contribute to making better predictions about the health of children resulting from embryos with the relevant mutations found in WGS-based screening. However, preconception testing of the prospective parents seems less useful for the interpretation of findings related to recessive disorders, given our observation that healthy persons may be compound heterozygous or even homozygous for mutations in this category.

Advancing knowledge in genomics may enhance the clinical validity of WGS-based embryo testing, as more will be known about the influence of modifier and protective genes and possible epigenetic influences that may explain our findings (von Kanel *et al.*, 2013). Also, other projects, such as the 'Deciphering Developmental Disorders' (Firth and Wright, 2011) study, may contribute to identifying the genetic cause of disorders, making knowledge about the genome more complete. Although it can be expected that with increasing up-to-date knowledge about the relation between the genome and the phenotype it will become possible in the future to make better predictions about the health of children resulting from the transfer of an embryo with a specific genotype, we conclude

that at present the clinical validity of WGS-based embryo screening is limited.

Clinical utility

As the analytical and clinical validity are still insufficient, a necessary condition for the introduction of extended or comprehensive embryo screening ('suitable test') is not met. Clearly, this would adversely affect the *clinical utility* of the screening, as it would lead to discarding embryos that may well develop into healthy children. Some may still defend the rapid introduction of WGS-based screening, arguing that mutations will be found that are thought to be causative at least in some cases, and that non-transfer of embryos with such mutations may still be warranted. However, as the number of available good-quality embryos in an IVF cycle is limited, there may not be much scope for choosing a mutation-free embryo, and the option of a new cycle just to avoid a 'suspected' embryo may well be disproportional. Moreover, with the current state of the art, prospective parents would be faced with choices based on unreliable predictions about the health of the children they could have as a result of transferring this or that embryo. Overestimation of the predictive value of adverse findings of WGS-based embryo screening may lead to the couple remaining childless or to undermining of their confidence in the health of any children they may still decide to have. In addition to this, they may also be confronted with equally unreliable information suggesting that they themselves are carriers of a potentially severe disease.

Conclusion and final remarks

At present, the drawbacks of WGS-based embryo screening appear to outweigh the possible benefits for prospective parents, making the introduction of such screening in clinical practice unwarranted and at best premature. It may be that further scientific developments will lead to improving the predictive accuracy of WGS-based embryo screening. Although that would take away the drawback of decision-making based on unreliable information, it does not automatically follow that WGS-based screening would then be unproblematic.

As screening aimed at simultaneously excluding a more than a limited number of genetic risk factors would very soon run into the problem of leaving no embryo for transfer (Hens *et al.*, 2012), a possible approach would be to always select the embryo(s) with the best health profile (while maintaining a threshold of at least not transferring high-risk embryos). However, the clinical utility of that approach would depend on whether meaningful choices between embryos with various health profiles can indeed be made. To say the least it is not clear whether that is the case. And even if it were, the amount of relevant data and the fact that (where genetic susceptibilities are concerned) even reliable information would be about risks rather than certainties, the feasibility of well-considered decision-making would not be obvious. Moreover, whose decisions should this be? As it can be argued that both prospective parents (whose child it will be) and professionals (given their active involvement in the creation of the child) have a say in this matter, WGS-based screening may have the potential of leading to conflicts between those stakeholders. Last but not least, a difficult problem is that choosing the embryo with the best profile will inevitably mean that children are born for whom some health prospects might already be known. The ethical question here is whether the future child should

be allowed to decide for herself what she wants to know about her genome (Hens et al., 2013).

We conclude that even if current limitations in terms of analytical and clinical validity can be overcome, the notion of WGS-based embryo screening still raises some difficult questions. It is presently unclear whether these can be satisfactorily answered. A possible alternative approach with its own advantages and disadvantages (De Wert, 2009) would consist of preconception screening of the prospective parents followed by targeted PGD.

Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

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Authors' roles

All authors participated equally in study design. The list of severe disorders was generated by I.L. and R.W. Data analysis was performed by R.W. Input on the ethics debate was provided by K.H., G.d.W. and W.D. The manuscript was drafted by K.H. and R.W. All authors revised the manuscript and approved the final version. The work was supervised by J.A.

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Conflict of interest

None of the authors has any conflict of interest to declare

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