European Journal of Human Genetics (2002) 10, 591 – 598

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ARTICLE

Preimplantation genetic diagnosis for Huntington's disease with exclusion testing

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Huntington's disease is an autosomal dominant, late-onset disorder, for which the gene and the causative mutation have been known since 1993. Some at-risk patients choose for presymptomatic testing and can make reproductive choices accordingly. Others however, prefer not to know their carrier status, but may still wish to prevent the birth of a carrier child. For these patients, exclusion testing after prenatal sampling has been an option for many years. A disadvantage of this test is that unaffected pregnancies may be terminated if the parent at risk (50%) has not inherited the grandparental Huntington gene, leading to serious moral and ethical objections. As an alternative, preimplantation genetic diagnosis (PGD) on embryos obtained *in vitro* may be proposed, after which only embryos free of risk are replaced. Embryos can then be selected, either by the amplification of the CAG repeat in the embryos without communicating results to the patients (ie non-disclosure testing), which brings its own practical and moral problems, or exclusion testing. We describe here the first PGD cycles for exclusion testing for Huntington's disease in five couples. Three couples have had at least one PGD cycle so far. One pregnancy ensued and a healthy female baby was delivered.
European Journal of Human Genetics (2002) **10,** 591 – 598. doi:10.1038/sj.ejhg.5200865

Keywords: preimplantation genetic diagnosis; Huntington's disease; exclusion testing; single-cell PCR

Introduction

Huntington's disease (HD) is an autosomal dominant, late-onset disease, for which the gene and the causative mutation (an expansion of a CAG repeat in the Huntingtin gene) were characterised in 1993.[1] This allowed for younger patients at risk of developing HD to know their carrier status accurately, and if found to be a carrier, to opt for accurate prenatal diagnosis or other alternatives such as the use of donor gametes. Patients who did not wish to know their carrier status, but still wished to have children who were not carriers, could opt for prenatal exclusion testing instead of direct determination of the length of the CAG repeat. Preimplantation genetic diagnosis (PGD) is a very early form of prenatal diagnosis in which embryos obtained *in vitro* are analysed for genetic disease, followed by the transfer into the mother's womb of those embryos shown to be free of the genetic disease under consideration.[2] We have described PGD for HD based on the enzymatic amplification of the CAG triplet repeat causing the mutation[1] and have applied this test in 16 couples in whom one of the partners was known to carry the expanded allele after presymptomatic testing.[3] It has been suggested[4] that this test might also be used for patients who do not wish to know their carrier status, in which case it would be termed non-disclosure, to differentiate it from exclusion testing. One centre has applied this test with apparently good results:[5] embryos are analysed before implantation without revealing to the patient any detail of the course of the IVF/ICSI cycle, after which only embryos without the expansion are transferred. No termination of pregnancy would have to be carried out, and

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Received 5 February 2002; revised 13 May 2002; accepted 5 July 2002

the parent's carrier status will therefore not have to be revealed. However, implementation of this method presents with important practical and ethical problems. These include, eg the fact that some lab or ward staff would know the carrier status of the patient to whom this status might be accidentally divulged and the fact that non-carrier patients would have to go through absolutely unnecessary IVF and PGD cycles. Finally, a mock-transfer would have to be performed if no embryos were available for transfer, lest the patient would draw the conclusion that he or she was indeed a carrier.[3,6] Exclusion prenatal testing, in which a foetus is checked for the presence of an allele from the affected grandparent was described as early as 1987,[7] initially to avoid the birth of offspring at risk to persons at risk for whom definitive testing was not possible. The most important drawback of this method is that unaffected foetuses may be terminated. Because of the ethical and practical problems associated with non-disclosure preimplantation testing, we decided to opt for exclusion testing at the single-cell level, after five couples in whom one of the partners was at risk for HD were referred to us.

Materials and methods

Informativity testing

Four different polymorphic dinucleotide repeats flanking the HD gene were tested: first, the informativity of D4S126[8] (located downstream of the HD gene, lod score 28.1309 for Φ=0.0229 in males and lod score 16.1158 for Φ=0.0210 in females, GENATLAS) and D4S127[9] (upstream of the HD gene, lod score 35.4987 for a Φ=0,0139 in males and lod score 36.3858 for a Φ=0,000 in females, GENATLAS) was tested. If these two markers were informative, no other markers were tested. If only D4S126 was informative, two other markers (D4S136[10] and D4S182[11]) also located upstream of the HD gene, were tested. These four markers are the only ones that could be used for PGD, since there are no other markers linked to HD that can be analysed using PCR. PCRs for these four different markers were performed as described in Table 1. The Expand High Fidelity kit (Roche) was used in all cases. One of the primers was fluorescently labelled and the fragment lengths were analysed on an ALFExpress Automated Sequencer (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) using Allelelinks software provided by the manufacturer. In each case, the couple and the parents of the spouse at-risk (both if available) were tested. If the spouse not at risk was also heterozygous, his or her parents were also tested, to establish phase.

Patient description

In our first patient couple (age of the wife at first PGD: 33 years), the mother of the husband at-risk had died of HD. In a preliminary molecular evaluation, the couple was shown to be informative for D4S126 and D4S127 flanking the HD gene (Figure 1). A first twin pregnancy, obtained after IVF because of concurrent infertility, was evaluated using these markers after chorionic villus sampling (CVS) and one foetus was shown to be at high risk, while the

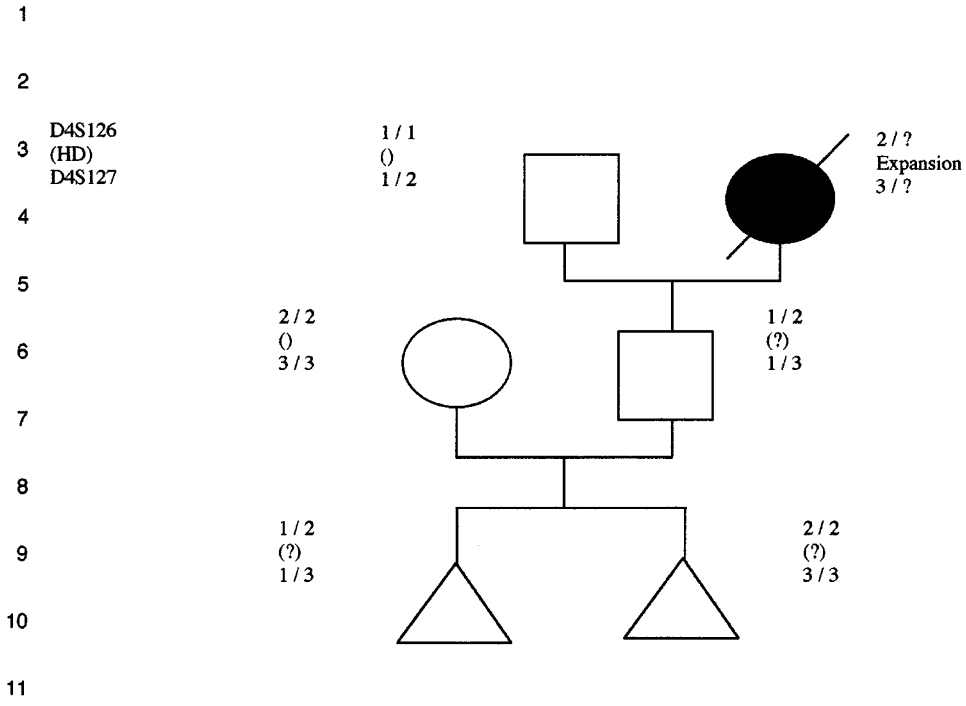

Figure 1 Pedigree with alleles for D4S126 and D4S127 in patient 1, with two possible combinations in the offspring.

second foetus was not. An attempt to terminate selectively the high-risk foetus led to the loss of both foetuses.

In our second patient couple, the husband came from a large extended family with several HD patients, including his father. The wife (33 years at first PGD) had undergone one prenatal diagnosis with exclusion testing after a spontaneous pregnancy, which was terminated because the foetus was at high risk. This couple was informative for D4S126 only and accepted PGD with only one marker, followed by control prenatal diagnosis using more markers in a possible pregnancy.

In the third couple, the wife (40 years at first PGD) was at risk since her father was diagnosed with HD at 48 years of age. Because of concurrent infertility, this couple initially opted for IVF combined with prenatal exclusion testing because preimplantation exclusion testing could not be offered at the time. No pregnancy ensued after one IVF treatment and one stimulation followed by insemination instead of oocyte pick up and IVF because of a poor response. Once preimplantation exclusion testing could be offered, and since the couple were informative for both D4S126 and D4S127, the couple opted for this solution.

In the fourth couple, the wife (27 years at first PGD) was at risk because HD had been diagnosed in her father. This younger couple chose preimplantation exclusion testing because they have objections to termination of pregnancy (TOP), although they are only informative for D4S127. They were counselled to undergo control prenatal diagnosis if a pregnancy ensued, but decided to decline this offer.

Finally, the fifth couple (age of the wife at first PGD consultation 32 years) where the husband was at risk was informative for D4S126 and 50% informative for D4S127. Because they objected to TOP, they had never attempted to become pregnant.

All patients had extensive genetic and psychological counselling concerning their options as persons at-risk to be HD carriers. Once their decision not to have presymptomatic testing was reached and they opted for HD exclusion testing and PGD, they were counselled at our own centre concerning the course and success rates of PGD.

Sampling of single lymphoblasts

Because the husband of the first couple was a double heterozygote for both markers, his lymphocytes were Epstein-Barr-virus transformed[12] to serve as a model for the development of single cell PCR. It is important to check heterozygous cells when performing single cell PCR, to check for allele drop out (ADO), ie the non-amplification of one allele. Briefly, single cells were sampled as follows:[3] a number of lymphoblast colonies were washed three times with PBS. Single cells were then washed three times in 2 μl drops of Ca^{2+}- and Mg^{2+}- free medium in a Petri-dish using fine hand-drawn micro-capillaries. They were then transferred blindly to 2.5 μl alkaline lysis buffer (ALB, 200 mM NaOH, 50 mM DTT) in 200 μl PCR tubes. Per three single cells collected, one aliquot from the last washing droplet was transferred to a PCR tube to serve as a blank. The samples were kept at -80°C until further processing.

Intracytoplasmic sperm injection (ICSI) procedure

ICSI was used to prevent contamination with sperm, which sticks to the zona pellucida after IVF and might therefore get into the PCR tube accidentally, and to avoid fertilisation failure.[13] The ICSI procedure was performed as described by De Vos *et al*.[14] Ovarian stimulation was carried out by a desensitising protocol of a gonadotrophin-releasing hormone agonist in association with human menopausal or recombinant gonadotrophins and human chorionic gonadotrophin. Cumulus-oocyte-complexes (COCs) were retrieved by vaginal ultrasound-guided puncture of the ovarian follicles, 36 h after HCG administration. The cells of the cumulus and corona radiata were removed by the combination of an enzymatic and mechanical procedure. Only metaphase II oocytes were injected. The oocyte was immobilised by slight negative pressure exerted on the holding pipette. A single spermatozoon was injected into the ooplasm. The Petri dishes with the oocytes were incubated in 25 μl droplets of IVF-100, G1.2 and G2.2 culture media (Vitrolife, Brussels, Belgium) in an incubator at 37°C (Heraeus, Vander Heyden, Brussels, Belgium) and under 5% O_2, 5% CO_2 and 90% N_2. Fertilisation was assessed 16 to 18 h after ICSI by ascertainment of the presence of two pronuclei. Further development was evaluated in the morning of day 2 and again at day 3, when embryos were evaluated before biopsy. After biopsy, the embryos were transferred to G2.2 medium (Vitrolife, Brussels, Belgium) until transfer at day 4 or 5.

Blastomere biopsy of cleavage stage embryos

Because of an earlier misdiagnosis in an embryo where only one blastomere was available for diagnosis, our subsequent policy has been that PGD diagnoses would only be based on analysis in two blastomeres.[3] This means that only embryos containing seven cells or more on the morning of day three are considered suitable for biopsy. A non-contact, 1.48 μm diode laser system (Fertilase, MTM Medical Technologies Montreux, Switserland) was used to create a funnel-shaped hole in the zona pellucida. Two clearly nucleated blastomeres were then gently aspirated through the hole.[15] The biopsied blastomeres were each transferred under stereo-microscopic (100× enlargement) guidance to a 200 μl PCR tube containing 2.5 μl ALB. The blastomeres were kept at -80°C for at least 30 min before lysis.

Single-cell PCR for D4S126 and D4S127 in duplex

The cells were lysed by incubating them in ALB at 65°C for 10 min, just prior to PCR. For both primer sets (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) the forward primer was labelled fluorescently with Cy5 (Table

1). The Expand High Fidelity Kit (Roche) was used for the amplification reactions. For the duplex PCR of D4S126 and D4S127, a first PCR round of 10 cycles with both primer pairs was carried out, followed by two separate PCR rounds for the two primer pairs separately using the PCR product of the first round as template. The reaction mix for the first PCR contained 0.4 μM of each primer set, 200 μM dNTP's, 1X Expand High Fidelity Buffer (Roche), 20 mM Tricine pH 4.95 for a final volume of 25 μl per sample and was decontaminated with 1 U per sample of the restriction enzyme *Nla*III by incubating the mix for at least 3 h at 37°C, followed by the inactivation of *Nla*III by incubation at 65°C for at least 20 min. Once the cells were lysed, the DNA Polymerase mixture (2.6 U per sample) was added to the decontaminated reaction mix and added to the lysed cells to a total volume of 25 μl. The first PCR programme was as follows: 2 min 95°C, 10 cycles of 30 s 95°C, 30 s 60°C, 30 s 72°C, followed by an extension of 5 min at 72°C. Two reaction mixes were prepared which each contained only one of both primer pairs. The reaction mixes contained 0.4 μM primer, 200 μM dNTP's, 1X Expand High Fidelity Buffer (Roche) for a final volume of 25 μl per sample and were decontaminated as described for the first reaction mix. Per sample, two μl of the first PCR reaction were added to 23 μl of each reaction mix. The PCR programme for D4S126 was 2 min 95°C, followed by 37 cycles of 30 s 95°C, 30 s 60°C, 30 s 72°C and an elongation step of 5 min at 72°C. The PCR programme for D4S127 was 2 min 95°C, followed by 37 cycles of 30 s 95°C, 30 s 55°C, 30 s 72°C and an elongation step of 5 min at 72°C. PCR reactions were performed either on a GeneAmp PCR System 9600 or 2400 (Applied Biosystems, Lennik, Belgium). After the PCR, 3 μl of the PCR products of the second PCR reactions were mixed with 3 μl loading buffer (5 mg/ml Dextran Blue in deionized formamide), loaded on a 6% sequencing gel (Life Technologies, Belgium) and run on an ALFExpress Automated Sequencer (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). The results were analysed using Allelelinks^R software provided by the manufacturer. For the PCR amplifying D4S126 only, only one PCR round was performed. The blastomeres were collected in ALB as described above. The PCR mixture contained 0.4 μM primer, 200 μM dNTP's, 1X Expand High Fidelity Buffer (Roche) and 20 mM Tricine pH 4.95 for a final volume of 25 μl per sample and was decontaminated as described for the first reaction mix. The PCR programme used was 2 min 95°C, followed by 45 cycles of 30 s 95°C, 30 s 60°C, 30 s 72°C and an elongation step of 5 min at 72°C. The PCR machines that were used, as well as the fragment analysis that was performed, were as described for the duplex PCR.

Results

Informativity testing

Couples 1 and 3 were informative for D4126 as well as for D4S127. Couple 2 was informative for D4S126 only, while

Table 1 Description of PCR protocols used for informativity testing

Locus	*Primer sequences*	*Composition of the reaction mix*	*PCR programme*
D4S126	F:GGATCCTGTCACTGTACTCCAGCC* R:TGCTTAACCAGTTTGACCATGAGG	0,4 μM primer, 200 μM dNTPs, 1X Expand HF buffer 1, 1.4 U Enzyme mix, 100 ng DNA	2 min 94°C, (30 s 94°C, 30 s 60°C, 30 s 72°C)X35, 5 min 72°C
D4S127	F:CCTCTGTTTGCAATCCATTT* R:GTCCCTTGCATGCCCTGGCT	0,4 μM primer, 200 μM dNTPs, 1X Expand HF buffer 1, 1.4 U Enzyme mix, 100 ng DNA	2 min 94°C, (30 s 94°C, 30 s 55°C, 30 s 72°C)X35, 5 min 72°C
D4S182	F:GCCTTGGGGCAGGGGCCGGTGAGTA* R:TCTATGAATTTCAAGGTGGCCATCT	0,4 μM primer, 200 μM dNTPs, 1X Expand HF buffer 1, 1.4 U Enzyme mix, 100 ng DNA	2 min 94°C, (30 s 94°C, 30 s 55°C, 30 s 72°C)X30, 5 min 72°C
D4S136	F:CTGACTTGATCCAATCCAAAGGAAAG* R:TTGAACCTAGTAGGCGGAAGTTGCAC	0,4 μM primer, 200 μM dNTPs, 1X Expand HF buffer 1, 1.4 U Enzyme mix, 100 ng DNA	2 min 94°C, (30 s 94°C, 30 s 55°C, 30 s 72°C)X30, 5 min 72°C

*Primer fluorescently labelled with Cy5.

Table 2 Summary of the seven PGD cycles performed

Patient no.	*Cycle no.*	*Number of COCs*[a]	*Number of 2PNs*[b]	*Number of embryos analysed*	*No. of at low-risk embryos*	*No. of at high-risk embryos*	*No. of embryos without diagnosis*	*No. of embryos transferred*	*Pregnancy*	*No. of embryos frozen*
1	1	10	6	5	2	3	0	2	–	0
	2	5	3	0	0	0	0	0	–	0
	3	8	4	2	1	1	0	1	–	0
	4	20	11	10	5	5	0	2	+	0
2	1	13	6	4	1	3	0	0	–	0
3	1	5	4	2	1	1	0	1	–	1[c]
4	1	6	2	0	0	0	0	0	–	0
Totals	7	67	36	23	10	13	0	6	1	1

[a]COCs=cumulus oocyte complexes; [b]2PN=two-pronucleate stage indicating normal fertilisation; [c]One high-risk embryo was cryopreserved.

couple 4 was informative for D4S127 only. Couple 5 was fully informative for D4S126 but only 50% informative for D4S127. Couples 2 and 5 were not informative for D4S136 and D4S182.

Results on double heterozygote lymphoblasts
A total of 30 lymphoblasts were amplified for both markers. All of these showed amplification for marker D4S126 as well as for D4S127. One of the cells showed ADO for D4S126 (4%) while none showed ADO for D4S127. None of 10 blanks showed contamination.

PGD cycles
Patient 1 In the first cycle, 10 COCs were retrieved, all of which were in metaphase II (MII) and were injected. Five embryos had developed beyond the 7-cell stage on the morning of day three and were biopsied. All 10 blastomeres biopsied from five embryos showed amplification. Two embryos (no 3 and 6, both 5-cell at transfer) were diagnosed as at low risk and were transferred on day 4. Three embryos were diagnosed as at high risk and were transferred each in their totality to a PCR tube for confirmation of diagnosis. All three embryos were confirmed to be at high risk as diagnosed during PGD. The patient did not become pregnant after this first attempt. During the second PGD cycle, five COCs were retrieved and four were injected. None of the embryos showed normal development and no attempt at biopsy was made. During the third cycle, eight COCs were retrieved, five of which were injected. Two embryos were analysed, one of which was diagnosed as at low risk and transferred, while the other was diagnosed as at high risk. This was confirmed at re-analysis. No pregnancy followed. Finally, during the fourth cycle, 20 COCs were retrieved and 16 were injected. Ten embryos were analysed, with five showing the high-risk genotype and five the low-risk genotype. Figure 2 shows the results of the PGD for one high-risk and one low-risk embryo. Two embryos (one at the blastocyst stage and one compacted) were transferred on day 5, ensuing in a singleton pregnancy. The patient declined confirmatory control prenatal diagnosis. This pregnancy has led to the birth of a healthy female child.

Patient 2 This patient started four PGD stimulation cycles, which were all cancelled before oocyte pick-up because of a poor response. The couple was counselled to opt for insemination with donor sperm, which would have solved at once the problem of the difficult stimulation (as this patient is normally fertile) and of the possible transmission of HD through the husband. However, the patient was adamant that his sperm would be used, and thus the couple preferred oocyte donation. The wife's sister then donated oocytes in the last cycle. As the husband was at risk in this couple, PGD still had to be performed. Thirteen COCs were retrieved, 10 MII oocytes were injected and four embryos were analysed. Three were shown to be at high risk and one was shown to be at low risk. Unfortunately, this low-risk embryo did not develop further and was not suitable for transfer.

Patient 3 Five COCs were retrieved, all of which were injected. Two embryos were available for biopsy. One was shown to be at low risk and was transferred, while the second one was at high risk. This embryo was cryopreserved at the patient's request in view of a possible future presymptomatic testing. The patient was not certain whether she would not choose to have presymptomatic testing at a later moment. If presymptomatic testing would show that she is not a carrier, then this cryopreserved embryo would not be affected and could then be transferred in a later cycle. No pregnancy ensued.

Patient 4 Six COCs were retrieved, of which four were mature oocytes and were injected. Only two were normally fertilised, none of which cleaved further normally. No PGD was attempted.

Patient 5 is planned for the near future.

Discussion

We have shown here the feasibility and accuracy of exclusion testing for HD on preimplantation embryos. In particular, a duplex PCR with two flanking markers was developed to detect any crossing-over between the used markers and the HD gene. Duplex (or multiplex) PCR at the single-cell level has its own difficulties as was the case for the PCR described here: the ideal annealing temperatures of the two primer sets were quite different, thereby rendering duplex PCR in just one PCR round totally inefficient and inadequate. This is why a first PCR round of only 10 cycles was used, involving both primer pairs, after which a second PCR round was performed using each primer pair in a separate reaction. One technical drawback to exclusion testing with two flanking markers is that the patients have to be informative for the markers used and that linkage has to be inferred from the analysis of family members other than the affected parent if this parent is not available for analysis, as in the first family. An illustration of this is that two couples who came to our centres asking for exclusion testing on preimplantation embryos were informative for one marker only. The patients were therefore strongly advised to have a control prenatal diagnosis in any ensuing pregnancy to detect crossing over between the used marker and the HD gene by analysing other flanking markers, which are detected by Southern blot and thus not amenable to single-cell analysis. However, couple 4 declined to have a control prenatal diagnosis. Couple 5 was fully informative for D4S126 and 50% informative for D4S127, but the combination of the two markers ensures complete diagnosis and detection of possible recombination.

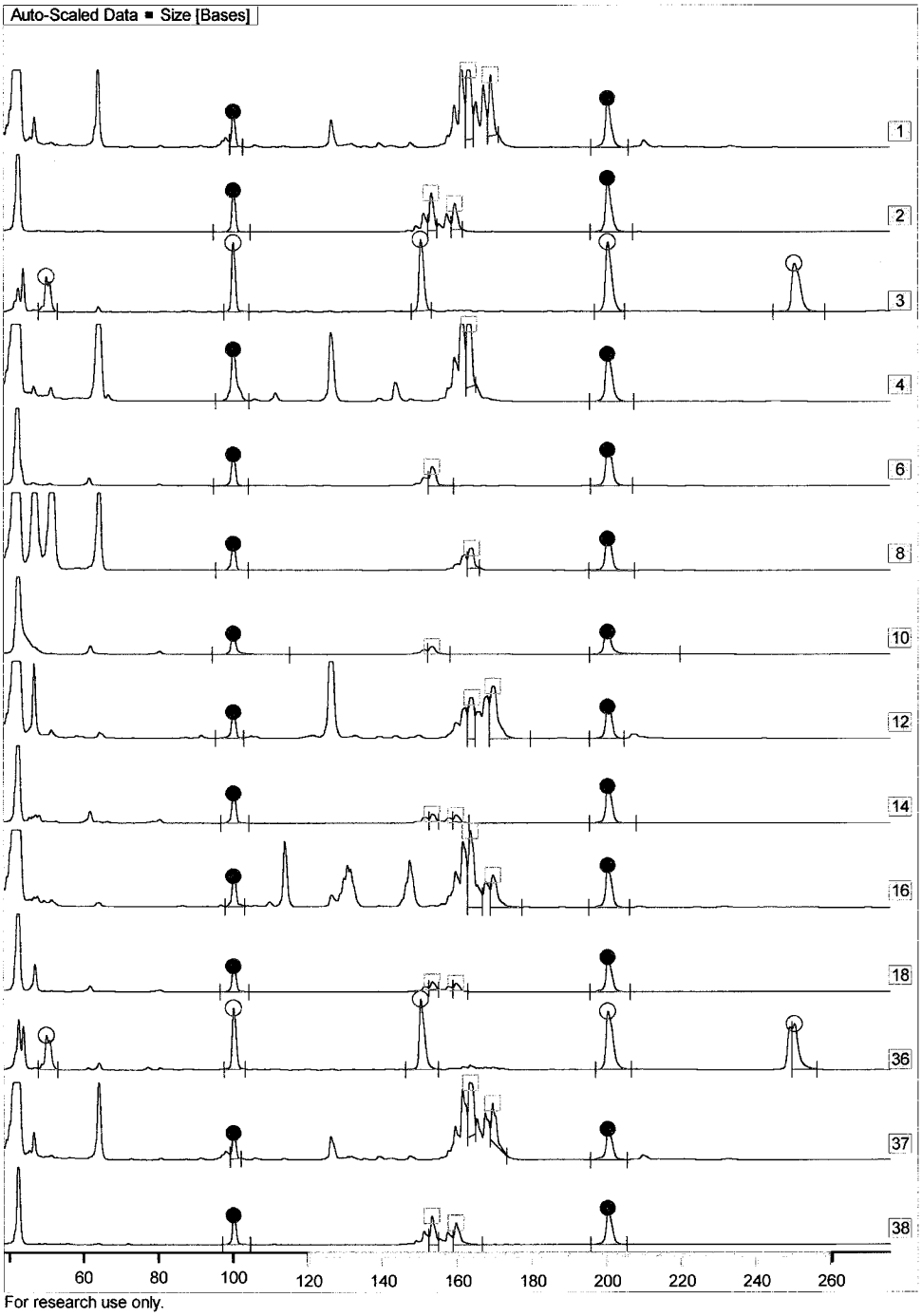

Figure 2 Example of a PGD result: lanes 1 and 37 show D4S126 (alleles 1 and 2) for the at-risk husband of couple 1; lanes 2 and 38 show D4S127 for the same patient (alleles 1 and 3). Lanes 3 and 36 show a 50 bp ladder (50, 100, 150, 200 and 250 bp marked with an open circle). Lanes 4 and 8 show D4S126 (alleles 2/2) for the two blastomeres of a high-risk embryo, lanes 6 and 10 show D4S127 (alleles 3/3) of that same embryo. Lanes 12 and 16 show D4S126 (alleles 1/2) for the two blastomeres of a low-risk embryo, lanes 14 and 18 show D4S127 (alleles 1/3) for the same embryo. Internal standards (100 and 200 bp) are marked with a solid circle.

The ethical implications are another important aspect of exclusion testing for HD. Exclusion testing was first proposed by Harper *et al*.[16] before the HD gene was cloned for couples who were not informative or for families where no material from affected members was available. Once direct testing of the CAG repeat was possi-

ble exclusion testing was used only for couples where the partner at risk did not wish to be tested. Even then, the uptake of pre-symptomatic testing remained low (19% in The Netherlands).[17] An important objection to exclusion testing is that the highest possible risk for the fetus is 50%. This is sometimes difficult to accept as reported by Tolmie *et al*.[18] In a series of 13 prenatal exclusion tests, four tested at low risk, and nine tested at high risk. Of these nine patients, three declined TOP, although all three patients had intended to go ahead with the TOP if an unfavourable result were obtained after PND. However, in other series the uptake of TOP in case of an unfavourable result was (nearly) 100%.[7,19,20] One consequence of not terminating a pregnancy at risk would be that, if the person at risk developed symptoms of HD, this would increase the risk to the child from 50 to nearly 100%. Moreover, the 'right not to know' of the child would be violated. Another point was raised by Maat-Kievit *et al*.[17] who stated that parents, after having used prenatal exclusion testing and if they are eventually found not to be a carrier or after staying asymptomatic into old age, could discover that TOP had been unnecessary. This is why eg in France, it is nearly impossible to receive the authorisation from a pluri-disciplinary PND centre for prenatal exclusion testing for HD. For all the above reasons, PGD for HD directly testing the embryos for the presence of the expansion, without informing the patients of the specific test results, was proposed as an alternative.[6] No information would be given to the couple that might provide a basis for inferring whether or not any embryos with the Huntington gene were ever identified. The authors went on to propose that this strategy could be used to reduce or even eliminate HD from the population. In a response to this, Evers-Kiebooms *et al*.[21] criticised the emphasis Schulman *et al*.[5] placed on the adverse effects of predictive testing and stress that counselling similar to the counselling given prior to predictive testing should be offered to patients opting for PGD. We are aware of one centre that has applied non-disclosure.[5] Eleven PGD cycles have resulted in four ongoing pregnancies. Possible problems with accidental disclosure of carrier status or other problems were not mentioned. After thoroughly discussing non-disclosure testing, it was decided at our centre to opt for exclusion testing. The main reasons for this were practical as well as ethical. Practical objections were: (1) in a large centre such as ours where many different professionals are involved in caring for the patients, ensuring non-divulgence of any information concerning the current PGD cycle, or even the carrier status of the patient itself, would be difficult. (2) If the patients are not pregnant after a number of cycles, they may conclude that they are indeed carriers. Ethical objections were: (1) after a number of cycles, and if no affected embryos were found, it would be reasonable to accept that the patient was not at risk. Continuing with a completely useless IVF treatment, which is not without adverse effects, would pose a serious ethical problem. It can be argued that with PGD with exclusion testing, unnecessary IVF treatments are also performed for those patients that are not carriers. Here however, no one (including the persons performing the PGD) knows the exact carrier status of the patient, and the useless IVF treatments should be weighted against the TOP of unaffected fetuses. (2) If no embryos were available for transfer, a sham transfer would need to be carried out in order not to allow the patient to think he/she is a carrier. (3) One person performing the PGDs would be informed about the patient's carrier status. It was considered that the psychological burden on this single person would be too high. The fact that three patients have now had at least one PGD cycle, that one of the patients is now pregnant, and that two more patients will soon undergo PGD proves that preimplantation exclusion testing is a valid option for patients at risk of HD who do no wish to know their carrier status. The diagnostic tests we have developed for this are highly efficient and reliable, as shown by the fact that all tested embryos were diagnosed, and that re-testing of non-transferred embryos confirmed our diagnoses. The low pregnancy rate (1/5 cycles, or 20%), although lower than pregnancy rates expected in regular IVF, is well within the expected range for PGD.[22]

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