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# First live birth in Greece after blastocyst trophectoderm biopsy and preimplantation genetic testing for Holt-Oram Syndrome

Konstantinos A. Economou<sup>1</sup>, Chrysanthi Bili<sup>2</sup>, Lina Florentin<sup>2</sup>,  
Fotios Thymis<sup>1</sup>, Dimitrios Papanicolaou<sup>1</sup>

<sup>1</sup> Life Clinic, Assisted Reproduction Unit, 166 Ippokratous Street, 11471, Athens, Greece

<sup>2</sup> Alpha Lab S.A., Center of Molecular Biology and Cytogenetics, 11 Anastasiou G. Street, 11524, Athens, Greece

## Correspondence

Dr. Konstantinos A. Economou, Life Clinic, Assisted Reproduction Unit, 166 Ippokratous Street, 11471, Athens, Greece,  
Tel: +30 6977245904, E-mail: keconomou@gmail.com

## Abstract

Preimplantation genetic testing for monogenic/single-gene defects (PGT-M) is a well established tool in assisted reproduction. A couple, where the male was a carrier of the c.709C>T (p.Arg237Trp) pathogenic variant (autosomal dominant) of the exon 7 of TBX5 gene causing Holt-Oram syndrome (HOS) attended our clinic for PGT-M. The father and their first baby boy were both affected by HOS appearing syndactyly. Employing a strategy of preserving embryos after blastocyst trophectoderm biopsy by vitrification cryopreservation, we managed to screen 10 embryos collected from a single IVF cycle. Four embryos were found unaffected after preimplantation genetic analysis for the paternal pathogenic variant. The transfer of one normal blastocyst post-thaw resulted in a healthy and uneventful pregnancy and in the live birth of a male neonate on the 39th week of pregnancy. This is the first report of a live birth in Greece after blastocyst trophectoderm biopsy and preimplantation genetic analysis for Holt-Oram syndrome.

**Keywords:** Preimplantation genetic testing for monogenic/single-gene defects (PGT-M), Holt-Oram syndrome (HOS), blastocyst trophectoderm biopsy, in vitro fertilization

## Introduction

Conventional preimplantation genetic testing for monogenic/single-gene defects (PGT-M) is considered to be an alternative to prenatal diagnosis for the diagnosis in vitro and before the establishment of a

pregnancy, of single gene disorders<sup>1,2</sup>.

Holt-Oram syndrome (HOS, OMIM #142900) is a rare autosomal dominant multiple malformation syndrome characterized by high penetrance and variable expression of upper limb abnormalities, congenital

heart defects (CHD) and/or cardiac conduction abnormalities<sup>3,4</sup>. It is caused by a defect in the TBX5 gene, localized on chromosome 12q24.21. Sequence variants of TBX5 gene, a member of the T-box family of transcription factors, have been identified to affect function in 75% of HOS cases<sup>5</sup>. Most are truncated alterations that result in haploinsufficiency, but occasionally sequence variations can lead to extension of TBX5 protein<sup>6</sup>. Since under- and overexpression cause the same phenotype, TBX5 function is considered to be gene dosage sensitive<sup>7</sup>. The prevalence of HOS is estimated to be 0.95/100,000 births<sup>8</sup>.

Upper-limb malformations may be unilateral, bilateral symmetric, or bilateral asymmetric and can range from triphalangeal or absent thumb(s) to phocomelia<sup>4</sup>. Other upper limb malformations can include unequal arm length caused by aplasia or hypoplasia of the radius, fusion or anomalous development of the carpal and thenar bones, abnormal forearm pronation and supination, abnormal opposition of the thumb, sloping shoulders and restriction of shoulder joint movement.<sup>4</sup> An abnormal carpal bone is present in all affected individuals and could be the only evidence of the disease.

A congenital heart malformation is present in 75% of individuals with HOS, most commonly involving the septum. Atrial septal defect and ventricular septal defect may vary in number, size and location. Complex congenital heart malformations can also occur in individuals with HOS. Individuals affected by HOS with or without a congenital heart malformation are at risk for cardiac conduction disease<sup>4</sup>, which is associated with heart failure and cardiovascular mortality<sup>9</sup>.

We report a case of a couple where the father and their baby boy are affected by HOS due to a TBX5 gene pathogenic variant, both presenting syndactyly. The disease was discovered after the birth of their first baby boy who was born with more severe syndactyly. The couple visited our clinic seeking IVF and

PGT-M to prohibit the passing of the disease to the second offspring. We employed a strategy of a single IVF cycle. The embryos created underwent blastocyst trophectoderm biopsy and subsequent vitrification. The samples were dispatched to a genetics laboratory for PGT-M analysis. The transfer of one healthy embryo post PGT-M, resulted in a healthy uneventful pregnancy and the live birth of a healthy male infant on the 39th week of gestation. This is the first case in Greece of a live birth after blastocyst trophectoderm biopsy and PGT-M for Holt-Oram syndrome.

### **In Vitro Fertilization**

After initial consultation, genetic counseling was provided to the couple, biopsy procedures and embryological processes were explained and signed consents were obtained. For the IVF attempt the GnRH antagonist stimulation protocol<sup>10</sup> was used, according to clinic's instructions. Intra-cytoplasmic sperm injection (ICSI) was used in the IVF attempt as previously described<sup>11</sup> in order to avoid sample contamination with paternal sperm DNA.

### **Blastocyst trophectoderm biopsy**

All embryos created for PGT-M analysis were cultured to blastocyst stage. A novel blastocyst trophectoderm biopsy protocol employed, in order to maximize the available blastocyst number for biopsy as has been previously described<sup>12,13</sup>.

The biopsy procedure took place employing an Olympus IX71 microscope (Olympus, Japan), equipped with an Eppendorf TransferMan NK2 micromanipulation system (Eppendorf, Germany) and pneumatic pipette control system (SAS-SE, Research Instruments, UK).

On day five of development a small opening of about 10  $\mu\text{m}$  was created through the zona pellucida (ZP) of each tested embryo by applying three con-

secutive 0.2 msec pulses of a non-contact 1.48-micron diode laser system (Saturn 5 Active Laser System, Research Instruments Ltd, UK). A mechanical micromanipulation of each blastocyst with the biopsy pipette followed in order to cause an artificial shrinkage of the blastocyst. The biopsy pipette was next inserted from the hole through the ZP and five to eight blastomeres were aspirated from the trophectoderm area.

In order to avoid excessive laser use that could potentially affect the chromatin quality of the biopsied cells and also harm the rest of the embryo, only two laser pulses of 0.2 msec irradiation time each were applied at the cell junctions on the biopsied trophectoderm mass. The trophectoderm cells were then dissociated from the blastocyst by fine microsurgical movements and by pressing the biopsy pipette against the holding pipette (flicking method) with simultaneous application of negative pressure in the biopsy pipette. The technique resulted into five to eight trophectoderm cells being microsurgically biopsied from each blastocyst. Special attention was

paid to leave the inner cell mass of each biopsied blastocyst unharmed by the procedure.

### Embryo vitrification and thaw

All blastocysts biopsied for PGT-M analysis were vitrified right after the biopsy procedure employing the Kitazato vitrification protocol<sup>14</sup> (Kitazato Corporation, Japan). After PGT-M two healthy blastocysts in two different frozen embryo replacement cycles were thawed according to the same protocol (Kitazato Corporation, Japan) and were left in culture for at least two hours before embryo transfer.

### Sample preparation and PGT-M analysis

Biopsied cells were thoroughly washed in four microdroplets of sterile non-stick washing buffer (NWB) [Phosphate-Buffered Saline, without magnesium or calcium (Invitrogen Life Technologies, USA)] and transferred to sterile 0.2 ml polymerase chain reaction (PCR) tubes in 1 µl of clean NWB. All biop-

Table 1. Primer pairs used for the detection of TBX5 pathogenic variant and linked markers

REGION/PCR	PRIMER NAME	SEQUENCE
p.Arg237Trp	TBX5_EX7FOUT	5' TTGCTTCTTTTGGTTGCCAG 3'
	TBX5_EX7R	5' CTGCTGGCTTACCTGGGTAA 3'
	TBX5_EX7FIN	5' ATGTCCTGAGGTGGTCTTGC 3'
	SNAP_TBX5_R237W	5' TTTTATAATCCCTTTGCCAAAGGATTT 3'
	IVS2_CA FOUT	5' GCTGAGGCACGAGAATCACT 3'
	IVS2_CA R	5' GGAATGGAATTGCCTTATGGT 3'
	IVS2_CA FIN	VIC 5' TGAACCTGGGAGGCAGAG 3'
	D12S2396 F	5' CAGGGATACGCACACACGTA 3'
	D12S2396 ROUT	5' TGCCAATTCTACCTCTCTCCA 3'
	D12S2396 RIN	FAM 5' TGCCAATTCTACCTCTCTCCA 3'
	D12S2397 F	5' TGAATTGTACACTTTAGAAAGGGTGAG 3'
	D12S2397 ROUT	TGGTGGTAAAAGAGGGTTGC 3'
	D12S2397 RIN	FAM 5' GCCAGAAGGCAGAGAAAGTG 3'
	D12S1341 FOUT	5' CCGAGGCAGGAGAATCACT 3'
	D12S1341 R	5' TGAAGCCTGTTGCTTTCTTTT 3'
	D12S1341 FIN	FAM 5' GAGACGGAGGTTGCAGTGAG 3'
	D12S1602 F	5' GGGCAAGGATCAAGCTCACT 3'
	D12S1602 ROUT	CAAGAGTGTGACTATTGCTTGATCA 3'
	D12S1602 RIN	FAM 5'AGAAAAGTTGTTTCATCTCCCTGG 3'

sied samples were transferred for PGT-M analysis and diagnosis to Alpha Lab S.A., (Athens, Greece).

Confirmation of the genetic status of the couple was performed as previously described<sup>15</sup>. PCR amplification of exon 7 of the TBX5 gene was performed using the OUT and IN primer pairs listed in Table 1, using a hemi-nested approach. Short tandem repeat (STR) markers (Table 1), closely linked to the disease-causing gene TBX5, that were informative for the couple, were also included to avoid a possible misdiagnosis resulting from allele dropout (ADO). All primer pairs were designed using “primer 3 (v. 0.4.0)” program. The reliability of the protocol was evaluated before clinical application on single lymphocytes collected from both parents, as previously described<sup>15</sup>.

DNA analysis of blastomeres was carried out on the same day as biopsy. Before proceeding to multiplex PCR, cells were lysed by incubation at 37°C for 1h in 10µl of proteinase K/SDS buffer and at 95°C for 10 min for proteinase K inactivation.

For the diagnosis of the disorder a nested multiplex PCR assay was used. The first round of PCR, that contained the external primers for the amplification of the gene region of exon 7 containing the pathogenic variant [c.709C>T (p.Arg237Trp)] and STR markers linked to TBX gene region for ADO detection, was performed in a total volume of 50µl containing 3.5 mmol/l MgCl<sub>2</sub>, 200mmol/l of each dNTP (Roche Diagnostic, Italy), 2.5 IU AmpliTaq Polymerase (Hot Start Taq Qiagen) and 10 pmol of each outer primer pair. The program used consisted of 35 cycles of 30 sec at 95°C, 30 sec at 60°C, 1min at 72°C. Each round of PCR was preceded by an initial 4 min denaturation step at 96°C and followed by a final extension step of 10 min at 72°C.

The first round of multiplex PCR was followed by three separate second round PCRs, one for the exon and two multiplex PCRs for the STRs (PLEX1 and PLEX2 as listed in Table 1) using the inner primer

pairs. Same conditions were used, only this time each PCR consisted of 35 cycles for the exon and 22 cycles for the STRs. Fluorescent fragments were analysed by 20 min of capillary electrophoresis on an automatic DNA sequencer ABI 3500™ (Applied Biosystems). Pathogenic variant analysis was performed using the minisequencing method (SNAPshot), as previously reported<sup>16</sup> and the specific primer for the pathogenic variant (Table 1).

### Case Report

We report a case of a couple where the husband and the first baby boy were affected by HOS. The male partner was a carrier of the c.709C>T (p.Arg237Trp) pathogenic variant of the TBX5 gene. The specific pathogenic variant was assessed as having an increased pathogenicity score of 98% and affects a functionally important site of the protein (T-box domain, TBX5 DNA binding). The husband was diagnosed with mild syndactyly but their first baby son was diagnosed with HOS and more severe syndactyly where a surgical operation was necessary to correct the condition. After thorough genetic analysis and counselling they presented on 01/02/2023 in our clinic for IVF and PGT-M for a healthy second child. At the time of presentation the woman was 33 and the husband was 40 years of age.

In their scheduled IVF cycle in our clinic on 12/03/2023, 26 ova were collected, 22 were mature post-cumuli/corona cell-denudation and all 22 were fertilized after ICSI. Ten blastocysts were submitted to blastocyst trophoctoderm biopsy on days five and six of development.

The biopsied cells were transported to Alpha Lab S.A., Athens, Greece for PGT-M analysis. Four embryos were diagnosed as unaffected for HOS (Table 2). All four were at blastocyst stage with qualities 4AA, 5BB, 5BB and 5BB according to Gardner’s blastocyst scoring criteria<sup>17</sup>.

Table 2. Results of PGT-M analysis for the paternal c.709C&gt;T (p.Arg237Trp) pathogenic variant of the TBX5 gene causing Holt-Oram Syndrome

Embryo Number	Blastocyst Quality	Day of Paternal Embryonic Development	Pathogenic variant c.709C>T (p.Arg237Trp)	Final Embryo Interpretation
1	5AA	5	Detected	Affected
2*	4AA*	5*	Not Detected*	Unaffected*
3	4AA	5	Detected	Affected
4	3BB	5	Detected	Affected
5	5BB	6	Detected	Affected
6**	5BB**	6**	Not Detected**	Unaffected**
7	5BB	6	Detected	Affected
8	5BB	6	Not Detected	Unaffected
9	5BB	6	Not Detected	Unaffected
10	5BB	6	Detected	Affected

\*Unaffected embryo transferred to the patient with negative result. \*\* Unaffected embryo transferred to the patient with positive result.

On 26/05/2023 the couple presented in our clinic for a frozen embryo replacement cycle. The 4AA blastocyst, unaffected by HOS (Table 2, embryo No 2), was thawed intact for the embryo transfer procedure. This frozen embryo replacement was unsuccessful and no pregnancy was achieved. On 15/11/2023 the couple attempted a second frozen embryo replacement cycle, where the 5BB unaffected blastocyst (Table 2, embryo No 6) was replaced. This attempt resulted in a positive outcome with a 365 mIU/ml initial  $\beta$ -human chorionic gonadotropin ( $\beta$ -HCG) result 10 days post-embryo transfer. Transvaginal ultrasound at eight weeks of gestation confirmed a clinical pregnancy identifying one endometrial sac with one positive fetal heart activity. On the 21st of July 2024 the patient underwent vaginal delivery and gave birth to a healthy male neonate at 39 weeks of gestation, weighing 4100 g and with an Apgar score of 10/10 without interurrences.

## Discussion

We report the first case in Greece of a Holt-Oram Syndrome PGT-M that resulted in the birth of a

healthy infant after blastocyst biopsy, vitrification and thaw of a healthy embryo used in embryo transfer. This live birth was achieved by applying trophectoderm biopsy on the embryos to be analyzed, on the sixth day of in vitro development. Blastocyst biopsy has now become the gold standard in order to obtain embryonic material for PGT-M purposes<sup>18</sup>.

The novel approach reported here is the direct hatching of the zona pellucida (ZP) and biopsy of the trophectoderm cells on day five or six of embryonic development, instead of a prior day three or day four ZP assisted hatching. This methodology has been reported to increase blastulation rate since the extra embryo manipulation taking place during day three or day four assisted hatching, may negatively affect embryo blastulation<sup>12</sup>. In addition the approach used here offers the advantage of the accurate positioning of the inner cell mass (ICM) of the blastocyst away of the area of micromanipulations. When an opening is created on day three there is a strong chance that on day 5 the embryo could be hatching from the ICM area. Performing micromanipulations in this case holds a high risk to traumatize or even damage the ICM, whereas in our approach, since the ICM has already been developed, the opening and the micro-

manipulations to remove the trophectoderm cells take place away from the ICM, keeping the latter unaffected and safe.

This is the first report in Greece of the live birth of a healthy infant after trophectoderm biopsy of blastocysts, vitrification and thaw of a biopsied embryo, in order to identify unaffected embryos by HOS suitable for embryo transfer. Two surplus healthy and good quality blastocysts (5BB and 5BB) remain in cryopreservation in our clinic for the study couple and could potentially lead to the birth of more healthy infants if transferred to the patient in the future.

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