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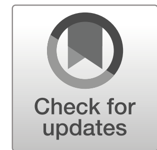

Reduced Foxo3a, FoxL2, and p27 mRNA expression in human ovarian tissue in premature ovarian insufficiency

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Abstract

Purpose Previous studies have suggested that deletion of Foxo3a, FoxL2, PTEN, p27, and AMH leads to early exhaustion of the primordial follicle pool and premature ovarian insufficiency (POI) in transgenic mice. Our aim was to assess for the first time, to our knowledge, messenger RNA (mRNA) expression of these genes and AMHR2 in human ovarian tissue from women with POI. We hypothesized that these genes would be underexpressed in POI women compared with healthy controls.

Methods mRNA levels were evaluated by quantitative reverse transcription-polymerase chain reaction and real-time polymerase chain reaction in cortical ovarian tissue obtained by laparoscopy from Caucasian Greek women with POI ($n = 5$) and healthy women with normal menstruation ($n = 6$). Morphological analysis of the ovarian biopsies was also performed to assess the presence of primordial or other types of growing follicles.

Results Ovarian tissue from POI patients showed lower Foxo3a, FoxL2, and p27 mRNA expression compared with controls ($p = 0.017$, $p = 0.017$, and $p = 0.030$, respectively). mRNA expression of AMH, PTEN, and AMHR2 was reduced in ovarian biopsies from POI patients as well. However, these differences were not statistically significant ($p = 0.143$, $p = 0.247$, and $p = 0.662$, respectively). Morphological analysis showed complete lack of follicular structures in all POI biopsies.

Conclusions Our findings suggest a possible role of Foxo3a, FoxL2, and p27 in the pathogenesis of human POI, which may prove to be of great diagnostic-therapeutic value. Further larger studies are needed to identify a similar pattern for AMH, PTEN, and AMHR2 and to investigate gene expression at a protein level.

Keywords Premature ovarian insufficiency · Primordial follicles · Foxo3a · FoxL2 · AMH

Introduction

Premature ovarian insufficiency (POI) is defined as the cessation of menstrual cycles and the loss of ovarian function in women younger than 40 years old [1]. It has been estimated that POI affects approximately 0.1% of women under the age of 30 and 1% of women under the age of 40 [2], with a significant impact on patients' physical well-being and subsequently on their emotional health and quality of life [3]. Women with POI not only experience vasomotor symptoms, but they also have increased risk for cardiovascular disease, osteoporosis, stroke, and even decreased age-specific life expectancy [4]. The main sequela, however, is infertility. Their chance of achieving a spontaneous pregnancy ranges between 4.4 and 10%, leaving them with limited fertility options [5, 6]. The etiology of POI, and especially its genetic background, has recently been at the center of scientific debate [7]. Possible causes include metabolic and autoimmune disorders, infections, and medical interventions, but POI can also be a phenotypic characteristic of various syndromes, like Turner's syndrome or blepharophimosis–ptosis–epicanthus inversus syndrome (BPES) type 1 [1, 3]. However, most cases of POI are idiopathic with an undoubtedly important genetic contribution, suggesting that there is still a lot to discover concerning its genetic origins [8].

Regardless of its exact etiology, overactivation and early depletion of the primordial follicles is one of the proposed mechanisms leading to POI [3, 9]. The female reproductive lifespan is determined by the existence of a set primordial follicle pool; premature exhaustion of this pool by a wide recruitment of the

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primordial follicles will eventually result in POI [9, 10]. Until the last decade, little was known about the exact factors that control the primordial to primary follicle transition, but it was thought to be regulated by intraovarian paracrine and autocrine molecules produced by the oocyte and the granulosa cells [11, 12]. The use of mouse knockout models revealed that certain genes, including forkhead box O3 (Foxo3a), forkhead box L2 (FoxL2), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), p27, and anti-Mullerian hormone (AMH), constrain follicular activation and maintain the primordial follicle reservoir [10, 13–19]. Loss of their inhibitory activity leads to accelerated activation of the primordial follicles and, subsequently, to premature depletion of the primordial follicle cohort and POI in genetically modified mice [14–19]. However, so far research has mainly been conducted in animal models and similar investigations in human material have been extremely limited.

The objective of our study was to assess for the first time to our knowledge, the messenger RNA (mRNA) expression of Foxo3a, FoxL2, PTEN, p27, and AMH and its type II receptor (AMHR2) genes in human cortical ovarian tissue obtained from women with POI compared with healthy women with normal ovarian reserve and menstruation. We hypothesized that these genes, which seem to inhibit the primordial to primary follicle transition, would be underexpressed in human POI ovaries.

Materials and methods

Participants

The study was conducted at the Gynecology Department of Patras University Hospital, Patras, Greece, between 2014 and 2017. All participants were Caucasian Greek in origin. Ovarian biopsy was performed in: (i) five patients with POI (POI, $n = 5$) and (ii) six control women with normal menstrual cycles (21 to 35 days), with a similar age distribution and treated during the same period (NM, $n = 6$). All POI patients were treated at the Division of Reproductive Endocrinology of Patras University Hospital and they had an established diagnosis prior to enrollment in the study. POI was defined as more than 4 months of amenorrhea, age < 40 years, hypoestrogenism, and serum follicle-stimulating hormone (FSH) > 40 mIU/L in two different measurements more than 4 weeks apart [20]. All POI cases were classified as idiopathic; the patients had normal karyotype and none of them had a history of chemotherapy, radiotherapy, infectious diseases, or metabolic or autoimmune disorders. Patients who had received any type of hormonal treatment during the 3 months preceding the procedure were also excluded. The control group consisted of women with normal menstruation, normal ovarian reserve assessed with AMH, and day 3 FSH and estradiol (E2) measurements.

Ovarian biopsy

Ovarian biopsy was performed during laparoscopic investigation for infertility and planned or acute laparoscopic pelvic surgery for benign disorders. The indications for laparoscopy were as follows: pelvic pain, adhesiolysis, and acute appendicitis for the POI group (one, two, and two patients, respectively) and pelvic pain, tubal patency test for infertility, and adhesiolysis for the control group (two, three, and one patients, respectively). All laparoscopies were negative for endometriosis. A single biopsy of a maximum size of 6–8 mm was sampled from each participant and, subsequently, was halved to allow both assessment of mRNA expression and morphological analysis. Cortical ovarian tissue was obtained from the surface of the antimesovarian edge. In control patients, the biopsy was obtained during the follicular phase of the menstrual cycle. If a dominant follicle was noted, then the biopsy was taken from the other ovary.

RNA extraction, complementary DNA synthesis, and quantitative real-time polymerase chain reaction

mRNA gene expression was studied through relative quantification in real-time polymerase chain reaction (PCR). The first half of each biopsy was preserved in RNAlater buffer and stored at − 80 °C until RNA extraction. Isolation of total RNA was carried out, including a 15-min DNAse I treatment, using the commercially available RNeasy Lipid Tissue Minikit provided by Qiagen (Qiagen, Germany), according to the manufacturer's protocol. RNA concentration and purity were estimated by UV spectroscopy. Complementary DNA (cDNA) synthesis was performed with a mixture of anchored-oligo(dT) primers and 1 μg of total RNA, according to the manufacturer's instructions (Transcriptor First Strand cDNA Synthesis Kit 04379012001; Roche Applied Science).

Real-time PCR was carried out in the LightCycler 2 Instrument (Roche) using the FastStart Universal SYBR Green Master (Roche Hellas). Table 1 presents the sequences of the PCR primers. PCR products were analyzed by the melting curve method, and relative expression was estimated by the $\Delta\Delta C_T$ method [21]. β-Actin was used as a housekeeping gene.

Morphological analysis of ovarian biopsies

The second half of each ovarian biopsy was placed in neutral formalin buffer (10%) for 48 h. Subsequently, it was embedded in paraffin blocks, sectioned at 5 μm, and stained with hematoxylin/eosin. The sections were examined to assess the presence of primordial or other types of growing follicles.

Table 1 Oligonucleotide primers used in quantitative RT-PCR (real-time polymerase chain reaction)

Gene	Forward primer	Reverse primer
FOXL2	5-ACTCGTACGTGGCGCTCAT-3	5-CTCGTTGAGGCTGAGGTTGT-3
FOXO3a	5-AATGAAAGCTCACTCTGGATTCC-3	5-TGTGCAATTCCTATGCAATC-3
p27	5-GTTAGCGGAGCAATGCGC-3	5-CAGGCTTCTTGGGCGTCTG-3
PTEN	5-CAAGATGATGTTTGAAACTATTCCAA TG-3	5-CCTTTAGCTGGCAGACCACA A-3
AMH	5-CGCCTGGTGGTCCTACAC-3	5- GAACCTCAGCGAGGGTGTT-3
AMHR2	5-CCCTACATCCCATCCACCT-3	5-GGTCTGCATCCCAACAGTCT-3

Hormonal measurements

Blood samples for hormonal determinations were obtained at admission. If needed, they were repeated during the 1st–4th day of the menstrual cycle in the healthy control women. FSH, luteinizing hormone (LH), and E2 were measured by chemiluminescence (Elecsys 2010; Roche Diagnostics). AMH was also measured by electrochemiluminescence immunoassay (Elecsys AMH Plus 2016; Roche Diagnostics). The detection range was 0.01–23 ng/mL, with a sensitivity of 0.006 ng/mL. The intra-assay and the inter-assay coefficients of variation for high values were 3.2 and 6.9%, respectively.

Statistical analysis

Data are presented as median and range (minimum-maximum). Non-normally distributed variables were compared using the nonparametric two-sided Mann–Whitney *U* test. *P* values < 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc).

Results

The clinical characteristics and the hormonal parameters of the women in the two groups are presented in Table 2. The median age was 26.5 years (23–32) and 27 years (22–33) for NM and POI women, respectively, while there was no difference in body mass index (BMI) between the two groups ($p = 0.329$). As expected, FSH and LH levels were significantly higher in POI than in control women ($p = 0.004$ and $p = 0.005$, respectively), whereas E2 and AMH levels were significantly lower ($p = 0.004$ and $p = 0.005$, respectively).

Table 2 Clinical and metabolic characteristics of control women with normal menstruation (NM) and women with POI

	NM ($n = 6$)	POI ($n = 5$)	*P* value
Age (years)	26.5 (23–32)	27 (22–33)	0.930
BMI (kg/m^2)	26.15 (19.8–30.1)	25.4 (19.7–28.7)	0.329
FSH (mIU/mL)	7.45 (3.6–14.4)	57.8 (48.6–104)	0.004*
LH (mIU/mL)	5.1 (2.4–11)	40.3 (33–80.2)	0.005*
E2 (pg/mL)	111.44 (29.4–210.4)	2.5 (0.9–9.4)	0.004*
AMH (ng/ml)	3.445 (2.49–6.37)	0.01 (0.01–0.02)	0.005*

Data are expressed as median and range (minimum–maximum). The Mann–Whitney *U* test was used to assess differences between the two groups

**P* values < 0.05 are statistically significant

Morphological analysis did not show evidence of any follicular structure in POI women. In contrast, all biopsies from the control women contained follicles at different developmental stages, from primordial to antral follicles.

Quantitative real-time PCR revealed a statistically significant 1.95-fold decrease in Foxo3a mRNA expression in ovarian tissue in women with POI compared with the healthy controls ($p = 0.017$) (Fig. 1a). Moreover, statistically significant underexpression of FoxL2 and p27 was demonstrated in the POI group compared with the control group (FoxL2 0.869-fold: $p = 0.017$; p27 0.799-fold: $p = 0.030$) (Fig. 1b, c).

As far as AMH, PTEN, and AMHR2 mRNA expressions are concerned, a relative reduction was found in ovarian tissue obtained from POI patients compared with the control subjects (1.103-, 0.66-, and 1.045-fold, respectively). However, none of these differences was statistically significant ($p = 0.143$, $p = 0.247$, and $p = 0.662$, respectively) (Fig. 1d–f, respectively).

Discussion

Our study showed that ovarian tissue from women with POI has reduced Foxo3a, FoxL2, and p27 mRNA expression compared with tissue from healthy women with normal menstruation, while it also demonstrates complete lack of follicular structures at histological examination. By contrast, no statistically significant alteration was identified for AMH, PTEN, and AMHRH2. To our knowledge, this is the first study so far to examine the expression of all six genes in human POI ovaries. Apart from McLaughlin et al. [22] and Carlsson et al. [23], all previous relevant research was mainly conducted in genetically modified mice. Instead of animal models of POI we used human cortical ovarian tissue from POI patients to

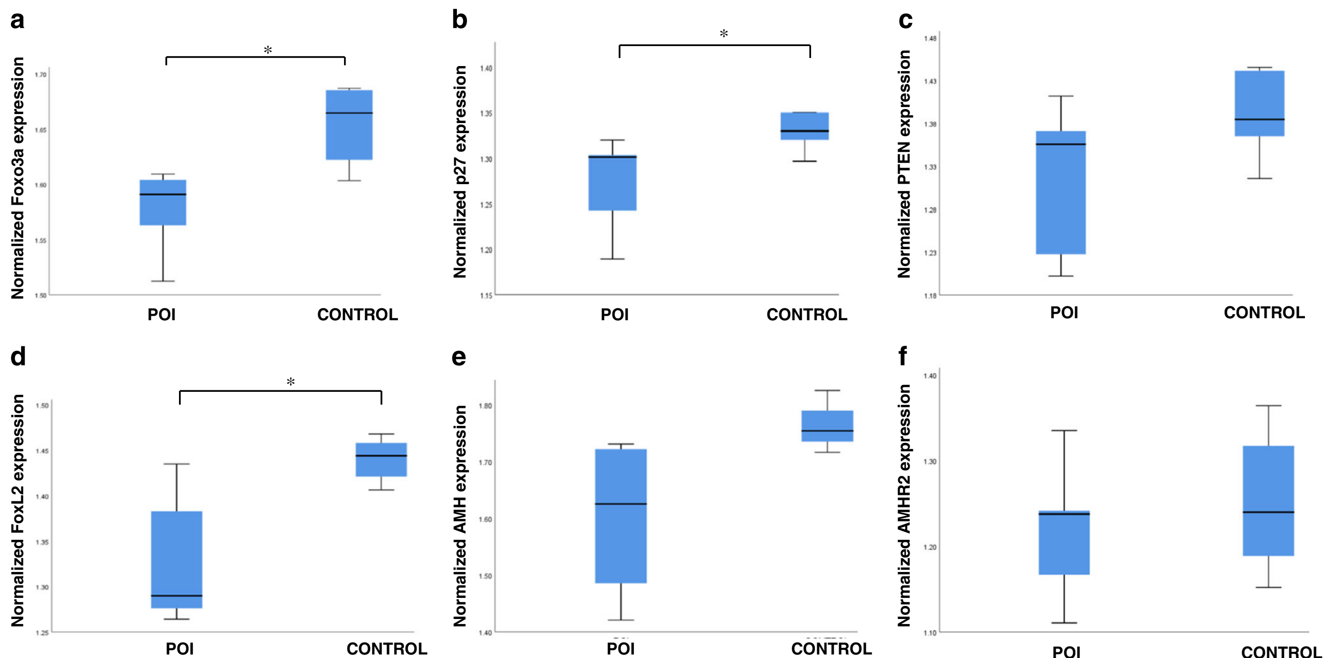

Fig. 1 Relative mRNA expression of Foxo3a (**a**), FoxL2 (**b**), p27 (**c**), AMH (**d**), PTEN (**e**), and AMHR2 (**f**) after normalization to the housekeeping gene β-actin in cortical ovarian tissue in POI and control women. Data are expressed as median and range (minimum–maximum). The Mann–Whitney *U* test was used to assess differences between the two groups. *P* values < 0.05 are statistically significant. *, statistically significant difference was observed in the relative mRNA expression of Foxo3a, FoxL2, and p27 between POI women and control women. **a** Foxo3a—POI group, 1.5909 (1.5122–1.6094); control group, 1.6647 (1.6035–1.6871), $p = 0.017$. **b** FoxL2—POI group, 1.2898 (1.2643–1.4351); control group, 1.4440 (1.4064–1.4680), $p = 0.017$. **c.** p27 - POI group: 1.3014 (1.1891- 1.3200), control group: 1.3299 (1.2968- 1.4520), p=0.030. Statistically insignificant difference was observed in the relative mRNA expression of AMH, PTEN, and AMHR2 between POI women and control women. **d** AMH—POI group, 1.6260 (1.4209–1.7315); control group, 1.7545 (1.7165–1.8258), $p = 0.143$. **e** PTEN—POI group,1.3558 (1.2020–1.4121); control group, 1.3848 (1.3159–1.4454), $p = 0.247$. **f** AMHR2—POI group, 1.2378 (1.1106–1.3353); control group, 1.2399 (1.1521–1.3643), $p = 0.662$

assess the expression of the genes that act as suppressors of follicular development.

The present findings regarding Foxo3a, FoxL2, and p27 gene expression are in accordance with data from previous animal studies, which revealed that loss of function of these three genes leads to global recruitment of primordial follicles and consequent sterility. Castrillon et al. [14] was the first to highlight the role of Foxo3a in maintaining the primordial follicle reservoir. Excessive activation and early depletion of primordial follicles was noted in Foxo3a knockout female mice, leading to infertility by the age of 15 months. In other words, Foxo3a null mice have a phenotype similar to human POI. His conclusions were supported by later studies by John et al. [15] and Pelosi et al. [24]. John et al. found that Foxo3a and not PTEN is the main regulator of primordial follicle activation through the phosphatidylinositol 3-kinase (PI3K) signaling pathway; the actual role of PTEN lies in regulation of Foxo3a expression [15]. Additionally, when a constitutively active form of Foxo3a was overexpressed in mutant mice, the ovarian reserve was preserved and a significantly higher number of primordial follicles were found at all ages after the second day after birth [24]. Despite the above findings in animal studies, Foxo3a has not to date been associated consistently with POI in humans; literature data regarding the correlation of Foxo3a mutations or polymorphisms and human POI are conflicting, and there is no consensus on the significance of Foxo3a variants in different ethnic groups [25–27]. Hence, Foxo3a underexpression in human POI ovaries is novel proof of Foxo3a's role as a regulator of follicular development in humans, while it also supports the concept that Foxo3a is involved in the pathogenetic mechanisms contributing to POI.

Consistent with studies in transgenic mice, we also demonstrated that human POI ovarian tissue underexpresses FoxL2. Two previous independent animal studies have shown that deletion of FoxL2 results in failure of granulosa cell differentiation, premature growth of the oocytes, and early exhaustion of the primordial follicle cohort [18, 28]. FoxL2 plays a key role in granulosa cell differentiation and quiescence of primordial follicles. FoxL2 knockout mice demonstrate premature activation of the primordial follicles, whereas at the same time, the granulosa cells are not functional due to an incomplete squamous to cuboidal transition, resulting in the absence of growing follicles, increased oocyte atresia, and infertility. Moreover, FoxL2 mutations have been described in cases of non-syndromic POI in humans [29], and they have been

identified as causes of BPES type I [30], an autosomal dominant genetic syndrome clinical manifestation of which includes POI. Taking the above into consideration, FoxL2 was expected to be underexpressed in human POI ovarian tissue.

As far as p27 is concerned, two different research groups have proposed that it down-regulates primordial follicle activation [17, 31], and this possibly happens independently of the PI3K–Foxo3a pathway [17]. In p27 knockout transgenic mice, the primordial follicle pool is exhausted prematurely, resulting in a POI phenotype in adulthood. However, the exact role of p27 in the pathogenesis of POI in humans is not yet well-established.

On the other hand, our hypothesis regarding AMH, PTEN, and AMHR2 underexpression in human POI ovaries was not confirmed by the results of the quantitative real-time PCR. We found a statistically insignificant reduction in the expression of these three genes at the mRNA expression level in human POI ovaries. These results are not in agreement with previous studies, which showed that inhibition of AMH and PTEN causes premature exhaustion of the primordial follicle reservoir and a POI phenotype [16, 19, 32–34]. AMH knockout mice are characterized by an accelerated recruitment of the primordial follicles [19]. Similar results were also reported by in vitro studies of cultured mouse [35] and human ovarian tissue [23], where exposure in AMH inhibited primordial to primary follicle transition. We still do not know exactly how AMH is implicated in the activation of primordial follicles, although a theory about possible inter-regulation between AMH and FoxL2 has recently been proposed [36]. Irrespective of the precise mechanism, experimental studies agree on AMH's role as an inhibitor of the primordial follicle recruitment. Similarly, PTEN's action as a negative regulator of PI3K has been investigated in experiments using human ovarian tissue in vitro, where McLaughlin et al. demonstrated that suppression of PTEN triggers activation of primordial follicles [22]. Interestingly, genetic studies in human POI populations have not revealed any causative mutation or single nucleotide polymorphism in the PTEN gene [37, 38], whereas conflicting results have been reported as regards the AMHR2 gene [39, 40].

These inconsistent findings could be partly explained by the main limitation of our study, which is the relatively small sample size. Inevitably, the number of participants was limited due to the low prevalence of POI and the undisputed difficulties in recruiting patients willing to proceed with an ovarian biopsy, and especially those suffering from POI. It is possible that the small sample size might contribute to some of the non-statistically significant results of our analysis. Another limitation is that the gene expression was explored at the mRNA expression level. The next step should be protein quantification. Techniques such as Western blot analysis or ELISA could have provided additional data regarding gene expression in the control women and patients with POI. They would also offer information about protein turnover as an additional regulation pathway. Considering the intricacies of the protein regulation process and the numerous post-translational modifications, the differences at the mRNA expression level might not translate into the protein level, a correlation that should be investigated in larger studies.

Despite the above limitations, our findings are novel and thus of high importance, especially given the lack of similar studies in the literature and how rare and valuable samples of ovarian tissue are. This is one of the few in the literature using human ovarian tissue and the only one, as far as we know, looking at mRNA expression of the above-mentioned genes. Even with that sample size, we managed to reveal statistically significant, albeit small, fold differences in the mRNA expression of Foxo3a, FoxL2, and p27. These changes reflect the altered follicular content in POI ovarian tissue, as demonstrated by the lack of primordial and growing follicles. Another explanation for the inconsistency between some of our results and the findings from experimental animal studies could lie in the complexity of the signaling pathways that control the activation of primordial follicles, with genetic alterations in different molecules (some of which still remain unknown), resulting in the same POI phenotype. Also, we should not disregard the fact that the so far available literature data are limited, they mainly originate from animal studies, and they have not been validated by research in human ovarian tissue and large genetic studies in human POI populations.

The exact mechanisms leading to POI are not clear as yet. As mentioned before, genetic variations causing overactivation of primordial follicles and premature depletion of their pool is one of the proposed theories. However, a POI phenotype may as well be attributable to constant underactivation/suppression of primordial follicle activation or their clearance directly from a dormant status [9, 10]. The use of mutant mouse models has expanded our knowledge regarding the factors that control initiation of growth of mammalian primordial follicles. It has now emerged that the highly coordinated activity of all inhibitors of follicular activation (Foxo3a, FoxL2, AMH, p27, and PTEN) is required to maintain the primordial follicle pool, whereas at the same time their survival is supported by other molecules, including 3-phosphoinositide-dependent protein kinase-1 (PDK1) and ribosomal protein S6 (rpS6) [41]. Not only has this growing body of research offered new insights into the genetic pathways of human POI, but it already has clinical applications. Future trends in fertility treatment for POI patients will probably include in vitro activation of primordial follicles, a method that has recently been tested with success [42, 43]. These molecules might also prove to be of great diagnostic value, addressing women who face a high risk of developing POI and could benefit from early fertility interventions.

In conclusion, for the first time, to the best of our knowledge, we have demonstrated that Foxo3a, FoxL2, and p27

mRNA expression is decreased in ovarian tissue obtained from women with POI, confirming the findings of previous experimental studies. The trends of our results can trigger further studies and they should be considered a first step in the exploration of the pathogenesis of human POI. Future larger studies are required to further validate our results, identify a possible similar pattern for AMH, PTEN, and AMHR2 and investigate the gene expression at a protein level.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Institutional Ethics Research Committee and Scientific Board of Patras University Hospital) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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