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ORIGINAL ARTICLE

Molecular study of human sperm RNA: Ropporin and CABYR in asthenozoospermia

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Abstract

Background Sperm motility is an essential aspect of human fertility. Sperm contain an abundance of transcripts, thought to be remnants of mRNA, which comprise a genetic fingerprint and can be considered a historic record of gene expression during spermatogenesis. The aberrant expression of numerous genes has been found to contribute to impaired sperm motility; these include ROPN1 (rhophilin associated tail protein 1), which encodes a component of the fibrous sheath of the mammalian sperm flagella, and CABYR (calcium-binding tyrosine-(Y)-phosphorylation-regulated protein), which plays an important role in calcium activation and modulation. The aim of this study was to investigate ROPN1 and CABYR gene co-expression in asthenozoospermic semen samples in comparison with normozoospermic samples.

Methods We studied 120 semen samples (60 normozoospermic and 60 asthenozoospermic) from Caucasian patients attending our centre for an andrological check-up. Total RNA was extracted from purified spermatozoa with RNeasy mini kit. ROPN1 and CABYR mRNA expression was analysed using RT-qPCR. Continuous variables were described as means ± standard deviations.

Results ROPN1 and CABYR mRNA were simultaneously downregulated in asthenozoospermic in comparison with normozoospermic samples. There was also a positive correlation between total progressive motility and ROPN1 and CABYR gene expression and between total motile sperm number and ROPN1 and CABYR gene expression.

Conclusions The results demonstrated downregulation of both ROPN1 and CABYR in asthenozoospermic samples and importantly, a positive correlation between the expression of the two genes, suggesting that ROPN1 and CABYR co-expression is a prerequisite for normal flagellar function and sperm motility.

Keywords Asthenozoospermia · CABYR · Ropporin · Human sperm mRNA

Introduction

Infertility can be defined as an inability to conceive after at least 12 months of regular unprotected sexual intercourse targeting the female partner's ovulatory period. It affects 15–20% of couples of reproductive age; in about one-third of cases, the cause is male factors, and in another third, a mix of male and female factors [1]. Various conditions can induce male infertility, including asthenozoospermia, i.e., reduced sperm motility. The World Health Organization (WHO) defines asthenozoospermia on the basis of a semen sample containing less than 40% of motile sperm and less than 32% with progressive motility (WHO 2010). Multiple factors may be involved in the onset of asthenozoospermia, and to understand them it is important to identify the role played by the genes involved in the sperm motility process [2].

Sperm contain an abundance of transcripts, thought to be remnants of messenger RNA (mRNA), which comprise a genetic fingerprint and can be considered a historic record of gene expression during spermatogenesis [3, 4]. It is thus possible that errors during the spermatogenetic process are encapsulated within the repository of spermatozoal RNA [5]. Despite its apparently inert transcriptional status, sperm contain a number of RNA molecules which can be

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transferred to the oocyte during fertilisation [6–8]. In recent years approximately 3000 different types of mRNA, small non-coding RNAs and large non-coding RNAs have been identified in sperm, although their functional significance is still unknown [9]. Not all RNAs found in sperm play a part during embryogenesis. It is therefore, important to understand if they are a mere relic of past gene activity or if they have an active role in post-fertilisation development.

In recent years, the scientific literature has attempted to identify any correlation between sperm RNA profile and male infertility, i.e., to establish if any useful information on an individual's fertility status can be found in the RNAs in his sperm. Various studies have shown that specific RNA molecules in sperm have a key role in conferring motility to sperm cells as well as preparing them for the capacitation and acrosomal reaction process [10, 11]. It was recently discovered that disrupted Ropporin and CABYR gene expression is associated with asthenozoospermia [2, 12].

Ropporin is a testis-specific gene found on chromosome 3 in location 3q21.1. It is expressed in round spermatids in the later stages of spermatogenesis. The gene's product, the protein Ropporin, is only found in sperm flagella. Ropporin is an integral part of the ibrous sheath (FS) of the sperm and, specifically, is located in the principle piece and end piece of the flagella [2, 13]. At the N-terminus it has a preserved sequence of 35 amino acids, with more than 50% homology to the regulatory subunit of type II cAMP-dependent protein kinase–RIIα [13, 14]. Through this sequence Ropporin binds A-kinase anchoring protein 3 (AKAP3), and this bond is regulated by phosphorylation events [15]. At the C-terminus, Ropporin has a PDZ domain involved in binding rhophilin, a testis-specific protein with a molecular mass of 70 kDa. Rhophilin interacts with the small GTPase Rho protein, which regulates cell adhesion to the substrate and cell movement through a complex modification of the actin making up the cytoskeleton [16, 17].

In 2011, Chen et al. [2] found that Ropporin was dependent on cAMP-dependent protein kinase and regulated sperm motility in mammals. Furthermore, its concentration was higher in sperm from normozoospermic samples than in sperm from asthenozoospermic samples.

Another protein which seems to have an important role in sperm motility is CABYR [12], which is coded by a gene located on the long arm of chromosome 18 in location 18q11.2. Like Ropporin, CABYR has an RIIα domain with which it interacts with AKAPs in the fibrous sheath of the sperm flagella [18–22]. CABYR is a testis-specific soluble polymorphic phosphoprotein, located in the sperm flagella, which binds calcium and is regulated by tyrosine, serine and threonine phosphorylation events during capacitation [23, 24]. It acts as both a calcium carrier and a scaffold for the enzyme complexes, which mediate energy production during the glycolytic process leading to hyperactivation of the sperm flagella. The calcium ion (Ca^{2+}) is thought to be a key regulator of correct sperm function and has a dual role: in the epididymis, it stimulates spermatid maturation and in seminal fluid it tends to inhibit sperm motility [25]. In addition, during capacitation, Ca^{2+} penetrates sperm in the female genital tract, contributing to hyperactivated sperm motility. The molecular event is an increase in the 86 kDa acid isoform of CABYR, which, after dephosphorylation, loses its ability to bind calcium [26].

The purpose of this study was to determine whether Ropporin and CABYR mRNA expression is associated with sperm function and to investigate the correlation between the expression of these two genes in asthenozoospermic semen samples.

Materials and methods

Patients

The study was approved by our University Hospital's Institutional Review Board. The written informed consent was obtained from all study participants. We studied 120 semen samples from Caucasian patients of similar ages (18–46 years) attending our Seminology Laboratory-Sperm Bank, "La Sapienza" University of Rome for an andrological check-up. The patients had not been medically or surgically treated in the 3 months prior to the study and did not have any conditions (fever, etc.) that might interfere with the semen analysis. All patients had normal hormone levels and karyotype. The samples were divided into two groups on the basis of their motility.

Semen analysis

All patients underwent semen examination. Semen samples were collected by masturbation directly into a sterile plastic container after 3–5 days of sexual abstinence. They were examined according to World Health Organization (WHO 2010) criteria. The following variables were taken into consideration: sperm concentration (N/mL), total sperm number (N/ejaculate), progressive motility (%) and morphology (% abnormal forms). In addition to raw motility data, absolute values in terms of millions of motile sperm per ejaculate were also calculated (obtained by multiplying the total sperm per ejaculate by the percentage of sperm motility). A sperm viability test was carried out to differentiate cell death from immotility by staining with eosin Y 0.5% in saline solution.

Osmotic shock

Semen samples were diluted with PBS to around 10×10^6 sperm/mL and underwent osmotic shock to eliminate the non-gamete cell component. Samples were incubated for 60 min at + 4 °C in cell lysis buffer (0.1% SDS, 0.5% Triton X-100 in distilled H_2O). After incubation, the absence of any round cells was confirmed under the optical microscope. The method used was as described in Paoli et al. [5].

RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA from purified spermatozoa was extracted with RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were determined using NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA).

cDNA reverse transcription of 100 ng of sperm total RNA was carried out for each sample using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions. Samples ($V_f = 20$ µL) were incubated for 10 min at 25 °C, for 120 min at 37 °C and for 5 min at 85 °C and were finally taken to + 4 °C, using the C-1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA).

To determine the expression of each mRNA, 2.5 µL of cDNA was added to 12.5 µL of 2X TaqMan Universal PCR Master Mix-No UNG (Applied Biosystems), 8.75 µL of RNase-free water (Ambion, USA) and 1.25 µL of 20× TaqMan gene expression assays (Applied Biosystems) specific for the gene of interest. qRT-PCR was carried out using Step One Real-Time PCR System (Applied Biosystems) as follows: denaturation at 95 °C for 10 min followed by 40 denaturation cycles at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Reverse and forward primers were as follows: Ropporin, forward primers 5′-GAA TGT GGG TCG CTT CAC G-3′, reverse primers 5′-TCC CCA TCC ACT TTG GCA ATA-5′; CABYR forward primers 5′-ACG GAA GCA GTT GGT GGT CT, reverse primers 5′-AAC ATC TGA GCA GCA AGC TGA G-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control; forward primer: 5′-TGC AGT GCC TAC CTT AAT GC-3′, reverse primer: 5′-CAC ATT GCA GCA CTT CCA TT-3′ [12, 32].

Units of fluorescence were converted automatically into cycle threshold (CT) values by ExpressionSuite v1.0.4 (Life Technologies, Applied Biosystems) and messenger RNA expression was calculated using the formula $2^{-\Delta\Delta CT}$ [27].

Statistical analysis

The statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS) 24.0 (SPSS Inc., Chicago, USA). Continuous variables were described as means ± standard deviations and medians. The Mann–Whitney test was used to compare the distribution of the variables. A p value of < 0.05 was considered as statistically significant. Spearman's correlation test was used to evaluate any correlations between expression of the genes considered and semen parameters.

Results

A total of 120 semen samples were divided into two groups based on their motility: Group A consisted of 60 samples with progressive motility ≥ 45% (normozoospermic samples) and Group B of 60 samples with progressive motility ≤ 30% (asthenozoospermic samples).

Table 1 shows the means, medians and SDs, semen parameters and sperm vitality for Groups A and B. There was no non-progressive motility in any sample; therefore, in this study, total sperm motility is equivalent to progressive motility.

Ropporin and CABYR expression in the two groups was analysed using RT-qPCR. Both genes were significantly downregulated (Ropporin: 22.9 times and CABYR: 7.1 times) in Group B in comparison with Group A ($p < 0.001$) (Fig. 1).

Gene expression was then analysed in relation to the percentage of total sperm motility, total number of motile sperm and total sperm number. In all groups, there was a statistically significant positive correlation between total progressive motility and Ropporin ($p < 0.001$, $r = 0.672$) and CABYR ($p < 0.001$, $r = 0.472$) gene expression (Fig. 2a, b),

Table 1 Means, SDs and medians of semen parameters and sperm viability for Groups A and B

	Volume (mL)	Sperm conc. ($\times 10^6$/mL)	Total sperm number ($\times 10^6$/mL)	Progressive motility (%)	Abnormal forms (%)	Viability (%)
Group A 60 pts	3.6 ± 1.5 (3.5)	125.1 ± 72.0 (112)	415.1 ± 194.3 (380)	58.1 ± 4.1 (60)	78.2 ± 3.2 (78)	81.0 ± 3.3 (80)
Group B 60 pts	3.7 ± 1.3 (3.7)	26.7 ± 25.1 (20)	96.9 ± 96.3 (64.4)	22.4 ± 7.1 (25)	84.3 ± 18.4 (90)	48.2 ± 19.3 (45)
p value	0.95	<0.001	<0.001	<0.001	<0.001	0.014

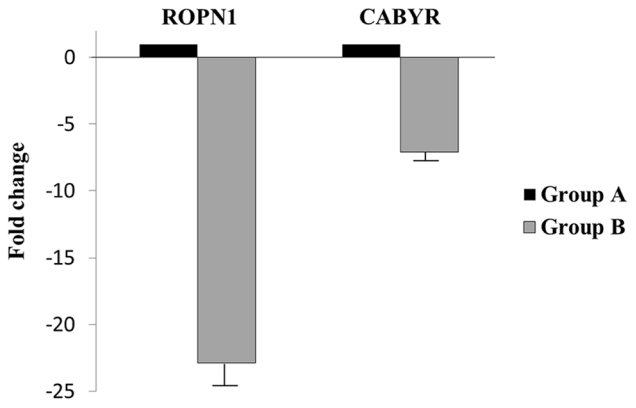

Fig. 1 Relative ROPN1 and CABYR expression in Group B vs. A, assuming Group A (control) = 1. Data are shown as mean ± standard deviation

between total motile spermatozoa and Ropporin ($p < 0.001$, $r = 0.601$) and CABYR ($p < 0.001$, $r = 0.486$) gene expression (Fig. 2c, d), and between total sperm number and Ropporin ($p < 0.001$, $r = 0.539$) and CABYR ($p < 0.001$, $r = 0.471$) gene expression (Fig. 2e, f). This demonstrated that there was a significantly higher quantity of Ropporin and CABYR mRNA in sperm from normozoospermic samples than from asthenozoospermic samples ($p < 0.001$). There was also a statistically significant correlation between the expression of Ropporin and of CABYR: $p < 0.001$; $r = 0.544$ (Fig. 2g).

Discussion

Asthenozoospermia is one of the most common sperm disorders in men, accounting for approximately 20% of cases of male infertility [28]. Despite numerous studies, the causes of this multifactor condition have not been fully established. Low sperm motility has been associated with reduced sperm genomic integrity, abnormal DNA condensation, the presence of antisperm antibodies [29], sperm midpiece defects, reduced acrosomal protein quantities, disruption of the calcium and cyclic adenosine monophosphate (cAMP)-dependent protein kinase pathways [3] and the impaired function of various genes. Asthenozoospermia was recently correlated with changes in the expression of Ropporin and CABYR, which encode two proteins located in the fibrous sheath (FS) of the sperm flagella [22].

The sperm flagella have four main pieces: the connecting piece adjacent to the head; the middle piece, containing a tightly packed helical array of mitochondria; the principal piece and the short end piece [17]. The main cytoskeletal structures are the axoneme, the outer dense fibre and the FS. The latter is the only cytoskeletal structure found in the sperm alone, and is believed to influence the degree of flexibility, the plane of flagellar motion and the shape of the flagellar beat [17, 31]. It also serves as a scaffold for both glycolytic enzymes and constituents of signalling cascades involved in the control of sperm maturation, motility, capacitation, hyperactivation, and acrosome reaction, which enables sperm to penetrate the zona pellucida [22]. To date, more than 20 proteins associated with the fibrous sheath of mammalian spermatozoa have been described [22], including AKAP3, AKAP4, Ropporin and CABYR.

Chen et al. [2] were the first to demonstrate Ropporin's role in regulating sperm motility. Using microarray analysis, the authors found that Ropporin was underexpressed in 32 asthenozoospermic semen samples in comparison with the same number of normozoospermic samples. In 2015, a study at the University of Cairo [32] investigated Ropporin gene expression in 24 asthenozoospermic samples from men with varicocele, both pre- and post-varicocelectomy. These authors found over-expression of Ropporin and an increase in sperm motility, concentration and normal forms following surgery. Finally, studies of double knockout mice (deficient in Ropporin 1 and ROPN-like protein) indicated impaired fibrous sheath integrity, a loss of AKAP3 from the flagella, sperm immotility and complete infertility [15].

The importance of CABYR was first revealed in a study which demonstrated its phosphorylation in the human sperm capacitation process [23]. Capacitation is a biochemical process which is essential for fertilisation and which takes place during the sperm's passage through the female genital tract [33]. Calcium is the key factor regulating sperm capacitation, hyperactivation and the acrosome reaction. At low intracellular Ca^{2+} concentrations the flagella beat symmetrically, while at higher concentrations (10–40 nM) the waveform becomes more asymmetric and sperm become hyperactivated [30]. CABYR's ability to bind calcium following phosphorylation events enables the generation, during glycolytic processes, of the energy leading to the hyperactivation of the sperm flagella [24].

In addition to its role in the capacitation process, the CABYR expression profile also suggests its possible role in the development of the FS. CABYR is expressed in the developing flagella during spermatogenesis up to the spermatid stage, when the FS structure is complete [34]. Young et al. [35] confirmed, in *Cabyr*-KO male mice, the importance of CABYR for the correct formation of the FS. Sperm from the *Cabyr*-KO mice presented significant disorganisation of the FS and an abnormal configuration of doublet microtubules, as well as reduced total progressive motility and fertility.

The involvement of CABYR in impaired sperm motility in humans was previously observed by Shen et al. [2], who demonstrated that, in comparison with normal human sperm, both the transcription and protein expression of

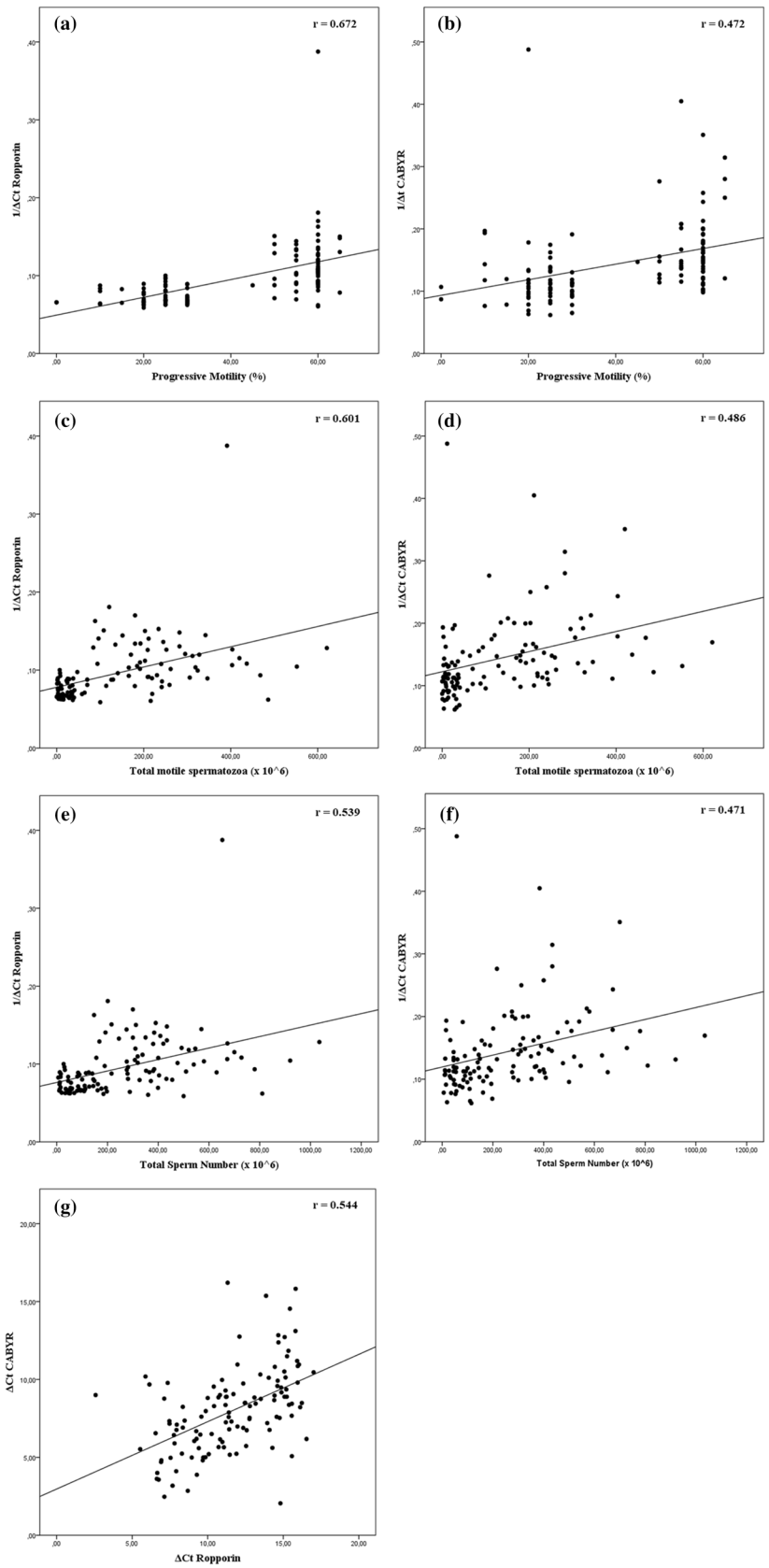

Fig. 2 Correlation between progressive sperm motility and Ropporin ($p < 0.001$) (**a**) and CABYR ($p < 0.001$) (**b**) gene expression. Correlation between total motile spermatozoa and Ropporin ($p < 0.001$) (**c**) and CABYR ($p < 0.001$) (**d**) gene expression. Correlation between total sperm number and Ropporin ($p < 0.001$) (**e**) and CABYR ($p < 0.001$) (**f**) gene expression. Correlation between Ropporin and CABYR ($p < 0.001$) gene expression (**g**). The reciprocal cycle threshold is plotted to display mRNA expression

CABYR was reduced in semen samples with idiopathic asthenozoospermia.

Ropporin is located near CABYR in the FS CABYR is found on the surface of the longitudinal column and ribs, while Ropporin is on the inner surface of the FS. The co-immunoprecipitation of Ropporin and CABYR has also been demonstrated, revealing a possible interaction between these two peptides [22].

In the present study, investigation of the Ropporin and CABYR expression profile in 60 normozoospermic and 60 asthenozoospermic semen samples suggested, in line with literature evidence, that their expression is related to sperm motility, as we found an abnormal expression of these genes in the asthenozoospermic samples, in which they were downregulated in comparison with the normozoospermic samples. Furthermore, the correlation we found between the expression of the two genes and the percentage of total number of motile sperm and total sperm number indicates that the quantity of Ropporin and CABYR mRNA in normozoospermic samples is significantly higher than in asthenozoospermic samples.

We also found a positive correlation between the expression of the two genes themselves, suggesting that they are inter-dependent and that their co-presence in sperm is a prerequisite for normal flagellar function and sperm motility.

Our results also showed that the reduced quantity of Ropporin and CABYR proteins found in other studies [2, 12] probably depends on common gene expression control mechanisms and not post-translation control, as we found a reduced quantity of both types of mRNA.

Conclusions

Our results show that CABYR and Ropporin are associated with impaired sperm motility. Their expression was reduced in asthenozoospermic samples in comparison with normozoospermic samples. Correlation analysis also revealed a direct correlation between the expression of the two genes. Therefore, our data demonstrate that Ropporin and CABYR co-expression is significantly lower in men with low sperm motility and is correlated with specific sperm function parameters.

The cause of Ropporin and CABYR downregulation in asthenozoospermic men is still unknown. It may depend on the action of transcription factors, the presence of polymorphisms or deletions in these genes, the action of microRNA, or epigenetic regulation mechanisms. As current treatments for severe oligoasthenozoospermia are not always effective, further investigation is needed to determine the reason for aberrant Ropporin and CABYR expression in idiopathic asthenozoospermic men. This information could contribute to understanding the molecular pathways of sperm motility and identifying clinical targets for future treatments such as germline gene therapy. In any case, these findings suggest that Ropporin and CABYR are important to spermatogenesis and could be useful biomarkers in the clinical diagnosis of idiopathic asthenozoospermia.

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Author contributions MM, DP, MP: Conceived of the study. MM, TC: Carried out the molecular studies. MP, MM: Drafted the article. DP, FL, AL: Gave final approval for the version to be published. FP, MP: Acquired and analysed the data. FL, AL: Revised the paper critically. All authors read, agreed upon and approved the final manuscript.

Compliance with ethical standards

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Conflict of interest None of the authors declares competing financial interests.

Ethical approval The study was approved by University Hospital's Institutional Review Board.

Informed consent All participants signed informed consent.

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