Are zona pellucida genes involved in recurrent oocyte lysis observed during in vitro fertilization?

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Abstract

Purpose Complete oocyte lysis in in vitro fertilization (IVF) is a rare event, but one against which we remain helpless. The recurrence of this phenomenon in some women in each of their IVF attempts, regardless of treatment, together with the results of animal experiments led us to investigate the possible involvement of the genes encoding for the glycoproteins constituting the zona pellucida (ZP).

Patients & methods Over the last ten years, during which we treated over 500 women each year, three women suffered recurrent oocyte lysis during their IVF attempts in our Centre for Reproductive Biology. For each of these three cases, we sequenced the four genes and promoter sequences encoding the glycoproteins of the ZP. The sequence variations likely to cause a change in protein expression or structure, were investigated in a control group of 35 women who underwent IVF without oocyte lysis and with normal rates of fertilization.

Capsule Recurrent oocyte lysis during in vitro fertilization does not seem to be due to any abnormality of zona pellucida genes

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Results & conclusion We found no mutations in the ZP genes sequenced. Only some polymorphisms present in the control group and in the general population were detected, excluding their specific involvement in the phenotype observed. Thus, although we suspected that complete oocyte lysis was due to a genetic cause, it did not seem possible to directly incriminate the genes encoding the proteins of the ZP in the observed phenotype. Further study of the genes involved in the processing and organization of ZP glycoproteins may allow elucidation of the mechanism underlying recurrent oocyte lysis during in vitro fertilization.

Keywords Zona pellucida · Oocyte lysis · Gene analysis · In vitro fertilization

Introduction

The zona pellucida (ZP) is a glycoprotein matrix surrounding oocytes and early embryos of mammals. It constitutes a species specific barrier [1] that plays a central role not only during the interaction between gametes but also after fertilization by preventing the risk of polyspermy [2]. ZP is also involved in the pre-implantation development of embryos. First, it maximizes the contacts between embryonic cells, promoting the compaction process [3]. Then, during the tubal transport, it protects the embryo against mechanical stress, prevents premature implantation, and may influence the axis of embryonic cleavage [4]. Moreover, the projections of follicular cells towards the oocyte establish direct connections through the ZP [5] allowing exchanges essential to folliculogenesis [6]. Abnormalities in the organization of the ZP could lead to oocyte-cumulus cell communication problems and thus affect oogenesis and oocyte quality [7].

In humans, the ZP consists of four distinct glycoproteins named zona pellucida sperm-binding proteins 1–4 (ZP1–4) [8, 9]. Biochemical analysis and electron microscopy studies have shown that the filaments constituting the ZP are formed

by the concatenation of the ZP2 and ZP3 heterodimers [2, 8, 10]. ZP1 homodimers stabilize the structure by linking together the assembly of the heterodimers [11, 12]; ZP1 and ZP4 are paralogs and may play similar roles [13].

This glycoprotein matrix is highly conserved among species, and the ZP polypeptides share several regions including a “zona domain”, a signal sequence, and a transmembrane domain preceded by a consensus furin cleavage site [14, 15]. The genes encoding these glycoproteins are both paralogs and orthologs, with a high level of identity between the genes as well as between different species.

In mice, the deletion of a gene encoding for one of the three murine ZP proteins results in the abnormal formation or even the absence of the ZP, leading to a more or less significant alteration of fertility depending on the gene involved [7, 16, 17].

In humans, the ZP1–4 proteins are encoded by four distinct genes *ZP1–4*, respectively located on chromosomes 11, 16, 7 and 1 [18–20]. The sequencing of these four genes has revealed the existence of polymorphisms related to heterogeneous morphological abnormalities of the ZP including oocyte lysis [21].

Oocyte lysis affects less than 5 % of the oocytes retrieved during in vitro fertilization (IVF) procedures [22]. Rare cases of oocyte lysis have been described in patients presenting a fragility of their overall oocyte cohort [23]. In our Centre for Reproductive Biology, three women who underwent treatment for infertility during the past ten years suffered from oocyte lysis of almost all their cohort at each of the IVF attempts. Cumulus-oocyte-complexes were effectively obtained after ovarian stimulation but the subsequent manipulations caused oocyte lysis, including the disruption of the ZP, circumventing the possibility of fertilization and embryonic development.

Here we report the sequencing in these three patients of the genes encoding the ZP1–4 glycoproteins and their promoter sequences. We use a control group of 35 women who underwent IVF without any evident ZP abnormalities and with satisfactory fertilization rates. We have tried to identify the sequence variations in these genes that may be involved in a structural defect of the ZP causing the phenotype of recurrent oocyte lysis. Our work was aimed at a better understanding of the mechanism underlying this phenotype, a phenomenon currently poorly known and which leads to potentially useless repeated treatments for infertility.

Patients and methods

Patients

Three patients, who had presented oocyte lysis during several attempts at in vitro fertilization, gave written informed consent for participation in this study. The research was approved by

the Ethics Committee of the University Hospital of Angers (DC-2011-1467). Table 1 summarizes the clinical and biological characteristics of the patients.

A control group was composed of 35 women who have benefited from IVF, without any evident ZP abnormalities and with satisfactory fertilization rates (over 25 %).

Long-agonist and antagonist protocols were used for the stimulation of ovulation. The long-agonist protocol included down-regulation with a GnRH agonist (Triptoreline: Decapeptyl® Ipsen Pharma, Boulogne Billancourt, France) followed by ovarian stimulation with FSH (Follitropine alpha: Gonal F® Serono, Switzerland; Follitropine beta: Puregon® Organon, Holland). The antagonist protocol involved ovarian stimulation with FSH followed by the administration of a GnRH antagonist (Cetrorelix: Cetrotide® Serono, Switzerland or Ganerelix: Orgalutran® Organon, Holland). The treatments were monitored by pelvic ultrasound examination and blood estradiol assays. Ovulation was induced by injecting human chorionic gonadotrophin (hCG) 5,000 UI, when at least three follicles larger than 17 mm were present, and the ovarian estradiol level was consistent. Oocytes were retrieved with a transvaginal probe 36 h after the HCG injection.

For each of the three women, we obtained several cumulus oocyte complexes in each cycle (Table 1). Patients 1 and 2 benefited from standard IVF and the setting of follicles was performed normally, but at the time of removal of the cumulus cells, the ZP appeared loose and fractured and no embryos were obtained; one of these women had been programmed for a second IVF attempt with intracytoplasmic sperm injection (ICSI), but oocyte lysis was observed immediately upon handling the oocyte. Patient 3 was directly proposed IVF with ICSI because her male partner suffered from oligo-asthenoterazoospermia. In the two ICSI attempts only one oocyte was injected in spite of the abnormal aspect of the ZP, which appeared to be fine and loose; an embryo was obtained and transferred in each case, but without resulting in a pregnancy.

Interestingly, two of the three women had a history of spontaneous pregnancy, and the third became naturally pregnant after the IVF attempts were stopped.

Screening of the *ZP1–4* genes

Blood samples were obtained from all women. DNA extraction was carried out using Blood Quick Pure kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. DNA qualification and quantification was performed using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

A total of 35 primer sets (Table 2) was used for amplifying the coding exons of the 4 ZP genes, including the exon-intron junctions. The duplication of exons 5 to 8 of *ZP3* on another locus, so called POM121 and *ZP3* fusion (*POMZP3*) [24], makes it difficult to interpret sequencing results. Like other

Table 1 Clinical and biological data of the three patients presenting repeated oocyte lysis during attempts at in vitro fertilization

Patient	Year of birth	Type of IVF attempt	Date of IVF attempt	Blockage	Stimulation	FSH units	Estradiol at release	Oocytes retrieved	Oocytes injected	Outcome	History of fertility
Patient 1	1977	cIVF	22/01/2008	A	P	2750	1953	18	–	Total lysis	Previous abortion 1996
		cIVF	26/06/2008	AN	P	2950	992	6	–	Total lysis	
Patient 2	1970	cIVF	29/03/2004	A	P	1350	970	4	–	Total lysis	Subsequent spontaneous pregnancy 2006
		cIVF	06/10/2004	A	P	2700	1716	6	–	Total lysis	
		ICSI	20/07/2005	A	G	2625	939	8	0	Total lysis	
Patient 3	1974	ICSI	07/03/2012	AN	P	1350	805	6	1	1 embryo transferred without pregnancy	Previous spontaneous pregnancies 1998 & 2001/(with a different man)
		ICSI + mild stimulation	01/10/2012	AN	P	750	477	3	1	1 embryo transferred without pregnancy	

A agonist protocol; AN antagonist protocol; G Gonalf®; P Puregon®; ICSI intracytoplasmic sperm injection; cIVF classical in vitro fertilization

authors [21, 25], we therefore studied only the first four exons of the variant encoding the longer isoform of *ZP3* (RefSeq: NM_001110354.1). In addition, we studied the promoter sequences, including the 5' untranslated region (5'UTR) of these genes, by sequencing the genes almost 800 bp upstream. Table 3 summarizes these features.

PCR reactions were carried out under standard conditions with 100 ng of genomic DNA in a 50 µl volume: 1.5 mM MgCl₂, 75 mM Tris–HCl (pH 9 at 25 °C), 20 mM (NH₄)₂SO₄, 0.01 % Tween 20, 50 pmol of each primer, 200 µM of each dNTP and 2 units of Hot GoldStar (Eurogentec, Seraing, Belgium) as follows: one cycle for 4 min at 94 °C followed by 30 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and one last cycle at 72 °C for 1 min. The PCR products were purified with the High Pure PCR Product Purification kit (Roche, Mannheim, Germany) and sequenced using a 3130XL DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The sequences obtained were compared with the reference sequences (Table 3) from RefSeq with Seqscape software v.2.6.0 (Applied Biosystems, Foster City, CA, USA) and manually checked. Each genomic variation detected was searched for in both the NCBI Database of Single Nucleotide Polymorphisms (dbSNP Build ID: 137) [26] and the Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: <http://evs.gs.washington.edu/EVS/>) accessed March, 2013.

The polymorphisms found, which could lead to abnormal expression or structure of the ZP proteins, were studied in the control group. Their allelic frequency (in this group) has been compared to the 1,000 Genomes reference female population [27].

Nomenclature of mutations

The *ZP1–4* mutations are described according to their respective reference sequences (Table 3). The nucleotide numbering of the nuclear genes reflects the cDNA numbering with +1

corresponding to the A of the ATG initiation translation codon in the reference sequence, according to the guidelines of the Human Genome Variation Society (<http://www.hgvs.org/mutnomen>). The initiation codon is numbered 1.

Results

No mutations, involving pathogenic or non-pathogenic sequence variations, common to the three patients or common to two of the three patients, were found in the genomic exons or exon-intron junctions.

We identified five exonic sequence variations in the three patients (Table 4). All the variations were located at sites known to be polymorphic and four of the six variations were heteroplasmic. Two of the heterozygous variations resulted in an amino acid change:

- 1) c.1078C>A located in exon 6 of *ZP1* with the consequence p.(Pro360Thr); the genotype C|C was referenced in the 1,000 Genomes female population [27] at a frequency of 98.8 % versus 1.2 % for genotype C|A; the sequencing of the area in 35 control patients showed a frequency of 2.8 % for genotype C|A (1 of 35 patients); and
- 2) c.91G>A in exon 1 of *ZP3* with the consequence p.(Gly31Arg); the genotype G|G was referenced in the 1,000 Genomes female population [27] at a frequency of 75.5 % versus 22.6 % for genotype G|A and 1.9 % for genotype A|A; the sequencing of the area in 35 control patients showed a frequency of 16.6 % for genotype G|A (6 of 35 patients).

In the *ZP2* promoter sequence (–73nt), we found a homozygous sequence variation in the three patients (Table 4). This genotype T|T was referenced in the 1,000 Genomes female population [27] at a frequency of 19.8 % versus 51.3 % for genotype C|T and 28.9 % for genotype C|C. The sequencing of the area in 35 control patients showed a frequency of 34.3 % for genotype T|T (12 of 35 patients).

Table 2 PCR oligonucleotides employed to evaluate *ZP1–4* mutations

Location		Forward primer sequence	Reverse primer sequence	Product size (bases)
<i>ZP1</i>	5' promoter	GGATGGTCAGGCGGTATAAG	CACAGGGTAACCCCAGGTC	993
	Exon 1	CTCCTCCATAAAAGGCCTGG	AGGGTCCCAGCTCCCTTC	334
	Exon 2	GAGACCCCAGCAACCTCTC	TCAGCCTGACATGACACTAGG	247
	Exon 3	CTGCCTGGGTATGCTAGGTG	GCCACCCTGTACTCCAGAAG	484
	Exon 4	TCCCAAGATTCAATTCCAGG	GGAGACAGCTCTCCTTCCG	284
	Exon 5–6	CGTCTCTGTGCTGGATGGAC	GCTCCGAAGCCTGTACTGAG	496
	Exon 7–8	AGTTTATTACGTAAACAGGATGCTC	GAGAAGAGAAGTCAGAGAGGGCTG	539
	Exon 9	CTGTCTAGACGGGTGAGTGC	GACCAGCTCCTCCCCTTAAC	280
	Exon 10–11	CCTTCCCTTTGTGCTTCCTG	TGTCAGCACAAATCTTGGGG	450
	Exon 12	TGTGATACAAAGTTCTGGGGTG	CAAATAACTTCTCCACACCTGG	279
<i>ZP2</i>	5' promoter	TGAGTGACTTTTGCCTTTCTC	CCTCCCTCCACTTCCAGAAAT	944
	Exon 1–2	ATGGGACAAGATTCAAGTGC	GAATGGAGGTTGTCTGAGCC	463
	Exon 3	TGAGAGGAGTCTATACACTTTTGTAGG	CCTAAGCCAAAGGCTGTCTG	220
	Exon 4	AGAAATGGACTGGTGGTCTTG	AGCCACTGCTTTACTCCAAAG	289
	Exon 5	TTTTCCCACTCAGATGGGTC	TGGTCTGATTTCTAAGCCCC	334
	Exon 6–7	TGTGCTTCTTCCACTGCATC	GCACTTAATAGGCATTAAAATGGG	605
	Exon 8–9	GCCCATTACTTGGATATATACTTGC	CAGCCGTATGAGGACTACCC	551
	Exon 10	AGTGGACTTGCTTCCTGCC	CATGTACAAGAGCCAAGGGAG	267
	Exon 11–12	CGTAGCATATCAGGTTGGGAG	TTTGGAGGGAAGGTAGGTACAG	530
	Exon 13–14	TGAGTTCAGGTGAGATAAAATTGC	ATGCTGCTCTAAAGCCCAAG	663
	Exon 15–16	GGCAGACATTGAATTTGACTCC	AAGAGCTCTGGGCAGTAAATAAAG	492
	Exon 17	TGACACACCTCTCGAACCCAC	ATGTAAGCACTGACCGCTTG	220
	Exon 18–19	ACCCCTCAGGGGACAGCTAAC	TGAGGAACAAAAGAAATGATCC	533
	5' promoter	CCCATGAAATTCCTCCTGTC	GGGGGTAGCACAGCTCAGTA	984
	Exon 0	AAGCCTCCTCCTCCAGCATT	TGCCCAAGGGTTGTAGATCCT	493
<i>ZP3</i>	Exon 1	AGCATCCCACGGGTATAAG	GTGGCCACAGTCCCCATAC	440
	Exon 2	GTATGGCTTGGGAGTACCCG	CATAGCCAGCCCCTTGC	259
	Exon 3	TGAGATACTCCCCATCAGTGG	GGGCAGGGGTCTGAGATG	244
	Exon 4	TTTTGGTTGGTTTGGTTGG	TGGCTTTAGGGTCATTTTGC	565
	Exon 5	CACCATGTTTGCCAGGCTAGT	CTGCTTGAAGGCCTCGCTAA	424
	Exon 6–7	CAGGTGATCTACCCGCTTG	GGACAATGAACAGCCTCTTGG	569
	Exon 8	CCAGTTCCCGTTAGGGACT	GACATGAACCCTGCCCTGTT	438
	5' promoter	AGGCAGAACCAGTTTGAGGA	AATCTGGTGCCTCAGGCTTA	934
<i>ZP4</i>	Exon 1	GCTCTTGGGAGAGGAGGC	CGGGAAGATAGGGAGACATC	309
	Exon 2–3	ATTGACAGTTGCTCCTTGGG	CACTGCACAGAGCAGGTCAG	501
	Exon 4	TCAATGACTTGTATCAAAATGGTATTC	TGGGTTTCATTGTAATTGCC	293
	Exon 5	TGGTTCTCCCCACAATGG	CAACATATTTCAGCTCTCCCC	328
	Exon 6	ACCAGATGCAAGTCAAGCAG	TTGTGGATTCAAGGGCTTACC	237
	Exon 7–8	AGGATGTGTAAGTACACCCAC	GACCAAGAGACCCAGCAGTC	629
	Exon 9	GGTTCTTTCATACACCTGTGC	CAGTTGTAAGTTGGGGAGGTTTC	279
	Exon 10	GGGAGGCAAGCAGGTTTAG	AGGAAAGGTTAGACATGGGAC	216
	Exon 11–12	AGAGACACTTGGCCTTGCAC	CCAAGCTTAAGAGATAAAGTGATGTG	619

Discussion

Since the ZP is closely involved in the mechanisms of oogenesis, fertilization and early embryonic development, any abnormality in the ZP may affect fertility.

Some morphological aspects of the ZP have been linked to human fertility. Thus, the birefringence of the ZP under polarized light during the IVF procedure has been associated with higher chances of pregnancy [28], and its thickness with better fertilization rates [29] and greater potential for

Table 3 Synopsis of the molecular study of *ZP1–4* genes

Gene	<i>ZP1</i>	<i>ZP2</i>	<i>ZP3</i>	<i>ZP4</i>
Cytogenetic location	11q12.2	16p12.2	7q11.23	1q43
Gene size (mRNA size)	8,150 bp (1,972 bp)	14,295 bp (2,266 bp)	17,117 bp (1,317 bp)	13,059 bp (2,474 bp)
Number of coding exons	12	19	8	12
Reference sequence (NCBI RefSeq)	NM_207341.2	NM_003460.1	NM_001110354.1	NM_021186.3
OMIM entry	*195000	*182888	*182889	*613514
PCR primer set (exons + promoter regions)	10+1	12+1	4+1	9+1

embryonic development [30–32]. Structural abnormalities of the ZP may account for the fragility observed during IVF in less than 5 % of the oocytes collected at the end of oocyte retrieval [22]. The increased incidence of fractured ZP in the oocyte cohort may reflect poor oocyte quality [33]. However, very few cases have been described with all the oocytes of a cohort showing fragility of the ZP [23, 34]. The occurrence of repeated oocyte lysis in different attempts for a same patient independently of the type of ovarian stimulation would seem to be specific to a given individual rather than due to an exogenous cause associated with the treatment or the environment.

Studies on knockout mice suggest that the origin of ZP abnormalities may be genetic, involving a mutation in the genes encoding for the ZP glycoproteins. Homozygous mice, with mutated *ZP2* or *ZP3* alleles, have oocytes devoid of the ZP, associated with impaired oocyte development, fewer oocytes ovulating, and abnormalities of the structure of the cumulus-oocyte complex, leading to infertility [7, 16, 35]. Heterozygous mice with a mutated *ZP3* allele, although naturally fertile, have oocytes in which the ZP is only half as thick as that of oocytes of wild mice [36]. *ZP1*-null mice, which are subfertile, have oocytes surrounded by a ZP matrix with various structural abnormalities, such as an accentuated perivitelline space and several ZP ghosts, indicative of oocyte lysis [17].

In human, ZP genes have been studied in cases of infertility associated with heterogeneous morphological abnormalities of the ZP. One study found certain polymorphisms, particularly in the *ZP2* and *ZP3* genes, significantly more frequently in patients with oocytes with anomalies such as an abnormally thin ZP, suggesting that the genetic background may play a role in the architecture of the ZP [21]. However, another study on three patients, all of whom had oocytes with abnormal aspects of the ZP, including an absence of the ZP, failed to find any genetic changes in ZP genes [25]. To our knowledge, our study is the first to focus on the specific phenotype of repeated oocyte lysis. This phenotype is reminiscent of the thinness and the fragility respectively found in the ZP of oocytes in *ZP3*^{+/-} and *ZP1*^{-/-} mice. Moreover, in mutant mice homozygous for *ZP1*, the administration of gonadotropins leads to laxity of the ZP [17]. Since hormonal treatment is known to modify the thickness of the ZP, the ovarian stimulation used in IVF probably leads to its increased fragility [37]. This may explain the spontaneous pregnancies of our three patients before or after the IVF attempts.

However, the analysis of the *ZP1*, *ZP2*, *ZP3*, and *ZP4* coding regions, including the intron-exon junctions (Table 3), failed to show any sequence variation common to the three women. Moreover, all the variations observed were polymorphisms previously described in the general population and were found at the same frequency in our controls, ruling out their implication in the oocyte lysis phenotype (Table 4).

Table 4 Molecular findings in the three patients presenting repeated oocyte lysis during attempts at in vitro fertilization

Location		Patient 1 genotype	Patient 2 genotype	Patient 3 genotype	Sequence variation	Consequence	rs#	Reference allele	MAF
<i>ZP1</i>	Exon 5	C T	C C	C C	c.858C>T	p.(=)	rs10897122	C	T=0.296
	Exon 6	C C	C C	C A	c.1078C>A	p.(Pro360Thr)	rs117566381	C	A=0.009
<i>ZP2</i>	5' UTR	T T	T T	T T	c.1-73G>T		rs2075521	G	T=0.446
	Exon 8	C C	T C	T C	c.747T>C	p.(=)	rs2075526	T	C=0.309
<i>ZP3</i>	Exon 1	G G	G A	G G	c.91G>A	p.(Gly31Arg)	rs2286428	G	A=0.131
<i>ZP4</i>	Exon 9	T T	T T	C C	c.1216T>C	p.(=)	rs35484380	T	C=0.001

5' UTR: 5' untranslated region; p.(=): protein was not analysed, but no change was expected; reference allele: allele in *Homo sapiens* (human) genome Build 37.3; MAF: global minor allele frequency, refers to the frequency at which the less common allele occurs in a global population; rs#: reference SNP ID number in the NCBI Database of Single Nucleotide Polymorphisms (dbSNP) [26]. MAF source: 1,000 Genomes [27]

We then focused on the 5'-flanking regions of the genes, which comprise the regulatory sequences for gene expression. In mice and humans, *ZP2* and *ZP3* promoters share TATAA boxes about 30 nucleotides upstream (−30 nt) of the transcription start sites, e-boxes (CANNTG) located at −200 nt, and short, highly conserved sequences, some of which are essential to the binding of transcription factors [38–40]. In patients with ZP abnormalities, a sequence variation at −87 nt has been found more frequently in the *ZP3* promoter sequence [21]. This sequence variation could reduce the activity of the gene by decreasing the production of the *ZP3* protein and altering the formation of the ZP, since the development of the ZP requires stoichiometric synthesis of the different glycoproteins [41]. We did not find this sequence variation in our three patients; however, we found a homozygous sequence variation at −73 nt in the *ZP2* promoter sequence in each of the patients. This genotype T/T is referenced at a frequency of 19.8 % in the general population. Moreover, the sequencing of the area in the 35 other patients who underwent IVF, without any evident oocyte abnormalities and with correct fertilisation rates, showed a T/T genotype frequency of 34 %. These findings rule out a link between this polymorphism and the phenotype studied.

Currently, little information concerning total oocyte lysis is available in the literature. The repeated occurrence of this phenomenon in women undergoing IVF is bound to cause considerable distress since no tangible explanation of the disorder can be offered. Interestingly, all our three patients had spontaneous pregnancies either before or after the IVF attempts. It may therefore be advisable in such cases to encourage recourse to natural cycles rather than to propose further stimulation attempts. The fact that total oocyte loss was observed consistently in all our three patients during IVF attempts, regardless of the procedure used, suggests a genetic aetiology. Our study, based on a small number of patients, needs to be substantiated by a multi-centre study, because of the low incidence of the phenotype. To date, there is no evidence of the implication of ZP genes in total oocyte lysis; it would be worth investigating the mechanisms underlying the processing and organization of the corresponding glycoproteins to elucidate the phenomenon.

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Contribution for each author

PMP, the principal investigator, takes primary responsibility for the paper. PMP, MF and PAB contributed to the conception, design and coordination of the research. CM and SL recruited the patients. PMP and MF contributed to the collection and analysis of data. PMP and MF contributed to writing the manuscript. PMP, MF, PAB, CM, VFL, SL, VP, PD and PR contributed to drafting the article and revising it, approving of the final version.