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Cell-free and intracellular nucleic acids: new non-invasive biomarkers to explore male infertility

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Abstract

Male infertility is a devastating problem that affects many couples worldwide. However, the molecular mechanisms and causes of idiopathic male infertility remain unclear. Circulating cell-free nucleic acids have an important role in human physiology and emerging evidence suggests that they play a role in male infertility. This review summarizes recent results on cell-free and intracellular nucleic acids in male infertility and discusses their potential use as biomarkers of male infertility in the clinical practice.

Keywords: cf-DNA, cf-mRNA, miRNA, piRNA, Male infertility, Seminal plasma, biomarkers

Résumé

L'infertilité masculine est un problème qui touche de nombreux couples. Cependant, aujourd'hui les mécanismes moléculaires et les causes de l'infertilité masculine idiopathique ne sont pas élucidés. Les acides nucléiques circulant ont un rôle important dans la physiologie et des évidences suggèrent qu'ils jouent un rôle dans l'infertilité masculine. L'objectif de cette revue est de mettre en avant les nouvelles avancées scientifiques sur les acides nucléiques circulant et non-circulant en lien avec l'infertilité masculine et de fournir une vue d'ensemble de leurs utilisation comme biomarqueurs en pratique clinique.

Motsclefs: cf-DNA, cf-mRNA, miRNA, piRNA, Infertilité masculine, Plasma séminal, Biomarqueurs

Background

Cell-free (cf) circulating nucleic acids in human plasma were first described by Mandel et al. in 1948 [1] and their importance as candidate biomarkers was recognized in the early 1990s after the study published by Sorenson et al. [2]. They include cell-free DNAs (cf-DNAs) and cell-free RNAs (cf-RNAs), which comprises messenger RNAs (mRNAs) and three major small non coding RNAs: microRNAs (miRNAs), piwi-interacting RNAs (piRNAs) and small interfering RNAs (siRNAs). Cf-DNAs are DNA fragments that circulate in the bloodstream following their release from apoptotic and/or necrotic cells [3]. They may also be actively secreted by living cells, for instance cancer cells. Therefore, circulating cf-DNA is a non-invasive source of material that can be used to collect genetic and epigenetic information on these cells [4, 5]. Cf-RNAs have been detected in many biological fluids. Like cf-DNA, they can be released by dying cells or be actively secreted by living cells. Therefore, they also may represent promising sources of material for assessing the gene expression profile of cells and tissues. Moreover, it has been demonstrated that extracellular small non coding RNAs may function as a signaling molecules in cell-cell communication [6, 7]. In body fluids, circulating cf-RNAs may be associated in protein complexes, such as density lipoproteins [8], with Argonaute

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2 proteins [9], with platelets in human plasma [10] or with microvesicles, such as exosomes that may participate in extracellular genetic exchange [6, 11, 12].

Circulating cell-free nucleic acids are found in and can be easily extracted from different biological fluids [12–14], such as serum or plasma [15], urine [16], seminal plasma [17] and semen [18]. They can be promising biomarkers to detect, monitor and predict the prognosis of several pathologies, particularly cancers, cardiovascular and neurological diseases [19–21]. Moreover, the study of fetal circulating nucleic acids in maternal plasma has opened a new avenue for the development of non-invasive prenatal diagnostic tools, as described by Lo et al. in 1997 [22]. In gynecological and obstetrical disorders, circulating cell free nucleic acids are considered to be potential biomarkers of female infertility [23, 24]. For instance, they can be used to detect ovarian reserve disorders, such as polycystic ovary syndrome [25], and to evaluate the quality of the follicular microenvironment [26].

Infertility is defined as the inability to achieve a clinical pregnancy after one year of regular and unprotected sexual intercourse. Worldwide, it affects approximately 15% of couples in age to procreate and trying to conceive [27, 28]. Female- and male-related causes explain each about one third of all infertility cases. The last third is due to both female and male causes or to no clear cause [29].

Reduced male fertility can be the result of congenital and acquired urogenital abnormalities, infections of the male accessory glands, increased scrotal temperature (varicocele), endocrine disturbances, genetic abnormalities (including chromosomal aberrations, polymorphisms, single or multiple gene mutations), mitochondrial dysfunction, or immunological factors and can be idiopathic in about 40% of patients [30–32].

In the last case, male infertility is defined as idiopathic when no casual factor is found and only semen analysis shows abnormalities, such as no detectable spermatozoa (azoospermia), decreased number of spermatozoa (oligozoospermia), decreased motility (asthenozoospermia) or abnormal morphology (teratozoospermia). When the last three abnormalities occur simultaneously, the syndrome can be described as moderate or severe oligoasthenoteratozoospermia [30, 32]. Epigenetic alterations should also be considered among the idiopathic male infertility etiologies because hyper- and hypo-methylation of imprinted and non-imprinted genes in spermatozoa have been associated with oligo-, astheno-, and/or teratozoospermia [33].

In clinical andrology and in assisted reproductive technology (ART), to document male infertility a detailed medical history should be obtained followed by a through clinical examination, semen analysis, endocrine and genetic evaluation and even testicular biopsy or puncture, to obtain an accurate diagnosis [31]. Most of the tests mentioned above are routinely used in the clinical practice however, these investigations mostly fail to elucidate the cause of male factor infertility. Moreover, these tests are cost- and labour-intensive (particularly, genetic and endocrinology tests), challenging and invasive (biopsy). For example, semen analysis alone is not reliable enough for accurate diagnosis and semen abnormalities do not exclude the possibility of normal fertility [34]. In addition, to diagnose the type of azoospermia a testicular biopsy is carried out to determine whether azoospermia is caused by a blockage (Obstructive Azoospermia, OA) or by primary testicular failure and germ cell loss (Non-Obstructive Azoospermia, NOA). These practices present a number of limitations and non-invasive biomarkers to determine the level of residual spermatogenesis in subfertile patients are necessary. On the other hand, as the efficiency of the existing hormonal treatements is rather low [32, 35], the identification of genetic factors that affect male infertility could provide valuable information for the development of targeted treatments. They could also help determining the causes of idiopathic infertility. In consequence, new non-invasive biomakers and more reliable diagnostics tools are required for discriminating among the different causes of infertility and/or for the diagnosis of subfertility in the clinical practice.

The aim of this review is to summarize the most recent scientific advance concerning cf-DNA, and circulating and intracellular small non-coding RNA and their potential use as non-invasive biomarkers.

Circulating DNA and male infertility

Cell-free DNA has been detected in human semen [17, 18]. Interestingly, its concentration in semen is much higher than in other body fluids [17]. As reported by Chou et al., cell-free seminal DNA (cfs-DNA) is associated with sperm parameters linked to normal sperm function such as velocity or morphology [18]. cfs-DNA level has been shown to be higher in seminal plasma of azoospermic than normozoospermic patients [17]. These observations suggest that cfs-DNA could be used in a search for biomarkers of sperm quality. On the other hand, it has been hypothesized that epigenetic alterations could cause male infertility..The presence of epigenetic information has been shown on cell-free seminal DNA [36]. This epigenetic information should reflect testicular epigenetic aberrations as semen is a mixture of secretions from the two testes, epididymes, seminal vesicles, bulbourethral glands and prostate [37]. Indeed, Wu et al. showed the existence of a correlation between methylation of specific promoters (such as CCNA1 and DMRT1) in cell-free seminal DNA and sperm physiopathology. In this study, the cfs-DNA methylation of these promoters was shown to be higher in the hypospermatogenesis group than in other groups with spermatogenic defects and normozoospermic patients [38]. These findings indicate that cell-free seminal DNA contains the epigenetic information of the male genital tract and could be a novel, non-invasive biomarker to detect spermatogenesis abnormalities.

Circulating and intracellular RNAs in male infertility

Besides cell-free DNA detection, cell-free RNA analysis presents theoretical advantages since it can provide valuable information on the gene expression patterns. In human semen, cf-RNA is a mix of transcripts derived from the male reproductive organs. It is fairly stable, particle-associated and more concentrated than in any other biological fluid [39, 40].

In this chapter, we will summarize the recent findings on cell-free mRNA, circulating and non-circulating small non-coding RNA related to male infertility.

Cell-free mRNA in male infertility

A recent integrated analysis of both mRNA and miRNA profiles in testis samples showed that many mRNAs and miRNAs are differentially expressed in patients with non-obstructive azoospermia (NOA) compared with patients with obstructive azoospermia (OA) or with controls (normozoospermia) [41]. This suggests that the two azoospermia forms (OA and NOA) may be distinguished at the transcriptional level. Similarly, studies on the potential of cell-free seminal mRNAs as new non-invasive biomarkers have shown that they could be used for the diagnosis of male infertility. For instance, in seminal plasma of NOA patients, the detection of the cell-free mRNA *DDX4* transcript (a germ cell-specific marker) was highly dependent on the presence of germ cells in testis. In contrast, the absence of the germ cell-specific (DDX4) RNA in cell-free seminal RNA was consistent with the histologic diagnostic of obstructive azoospermia. This finding suggests that cfs-mRNA DDX4 might be used to evaluate the type of azoospermia [42]. In addition, Pansa et al. showed that the germ cell-specific *ESX1* transcript is down-regulated in seminal plasma of patients with NOA compared with controls (normozoospermia). Moreover, they found that the change in expression is correlated with the severity of the spermatogenic defects [43]. Consequently, both *DDX4* and *ESX1* germ cell-specific mRNAs could be used as suitable molecular markers to predict the presence of residual spermatogenesis.

In conclusion, semen cf-RNA seems to be a promising non-invasive biomarker of the male reproductive system for diagnostic medicine and research due to its abundance, stability and the information it carries about the reproductive tract organs [40, 44].

MicroRNAs in male infertility

MiRNAs play a crucial role in numerous aspects of cell biology, such as cell differentiation, metabolism and apoptosis. They are small (about 22 nucleotide-long), non-coding RNAs that are evolutionarily conserved and that modulate gene expression post-transcriptionally by targeting specific mRNAs [45]. It has been shown that miRNAs can affect mRNA stability and in some cases influence protein synthesis through target cleavage and/or translation inhibition [45]. Human sperm contain a stable population of miRNAs related to embryogenesis [40] and to spermatogenesis [46, 47]. Their role in the control of male germ cell differentiation [48] highlights the fundamental contribution of these molecules to fertility regulation.

Salas-Huetos et al. listed 221 miRNAs that are consistently detected in spermatozoa of fertile men. Among the ten most expressed miRNAs, three directly control spermatogenesis because they are involved in the regulation of the E2F-pRB pathway during male meiosis (hsa-miR-34b-3p), in cell cycle progression (hsa-miR-132-3p) and in sperm differentiation (hsa-miR-191-5p). The others are linked to cancer and aging (hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-375, hsa-miR-19b-3p, hsa-miR-200c-3p), or to unknown biological functions (hsa-miR-891a, hsa-miR-1233-3p) (see Table 1) [46].

Some studies reported that the expression profiles of specific miRNA populations from tissues, cells and fluids are altered in males with different fertility problems (Table 1). Specifically, the first report showed that the testis miRNA expression pattern of patients with NOA is different from that of fertile males. The authors could identify 154 miRNAs that are down-regulated and 19 that are up-regulated in testis samples from patients with NOA compared with controls [49]. Among these differentially expressed miRNAs, there are several members of the hsa-miR-17-92 cluster (hsa-miR-1, hsa-miR-181a, hsa-miR-221 and hsa-miR-9*) and of the hsa-miR-371,2,3 cluster (hsa-miR-371, hsa-miR-372, hsa-miR-373) (see Table 1). MiRNAs from both clusters are downregulated in patients with NOA. Moreover, some of these miRNAs are potential novel oncogenes that might participate in the development of human testicular germ cell tumors ([49] and references therein). Then, Abu-Halima et al. showed that hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-449a and hsa-miR-449b, which are structurally similar, are down-regulated in testes of infertile men with different histopathologic patterns (the most common feature of male-factor infertility). This suggests that these miRNAs could contribute to regulate male germ and somatic cell development and that their alterations might be associated with reproductive abnormalities [50].

Table 1 List of miRNAs related to male idiopathic fertility disorders and their putative functions

Patients	Samples	miRNA expression	hsa-miRNA	Putative functions
Fertile (controls)	Spermatozoa	-	miR-34b-3p [46]	E2F-pRb regulation
		-	miR-132-3p [46]	Cell cycle progression
		-	miR-191-3p [46]	Sperm differentiation
		-	miR-30b-5p, miR-30c-5p, miR-375, miR-19b-3p, miR-200c-3p [46]	Cancer and aging
		-	miR-891a, miR-1233-3p [46]	Unknown biological function
NOA	Testis tissue	Down	miR-1, miR-181a, miR-221, miR-9*, miR-371, miR-372, miR-373 [49]	Development of human testis germ cell tumors
Idiopathic infertility (NOA, Asthenozoospermia, Oligozoospermia, Astheno-Oligozoospermia)	Seminal plasma, Spermatozoa, Extracellular Microvesicles,	Up	mir-429 [36, 48, 51, 52]	Spermatogenesis
			miR-1275 [47, 53]	
	Spermatozoa, Extracellular Microvesicles	Down	miR-15b [47, 48, 51, 53]	
	Spermatozoa, Seminal plasma	Up	miR-141 [36, 48, 51]	Embryonic development
	Spermatozoa	Up	miR-19b [47, 54], miR-483-5p [47, 53]	Unknown
	Spermatozoa	Down	miR-19a [48, 53], miR-1973 [48, 51]	Unknown
	Spermatozoa, Extracellular Microvesicles		miR-28-5p [47, 53]	
NOA	Seminal plasma, Spermatozoa, Testis tissue	Up	miR-429 [36, 48, 52]	Spermatogenesis
	Spermatozoa	Down	miR-34* [52], miR-34c-5p [48, 52, 55], miR-122 [48, 52, 55]	
Asthenozoospermia	Spermatozoa	Up	miR-27a [47, 57]	Signaling pathway
	Spermatozoa	Up	miR-548b-5p, miR548c-5p, miR-548d-5p [47]	
	Spermatozoa	Down	miR-34b-3p [47, 51]	Spermatogenesis
	Spermatozoa	Down	miR-520 h, miR-520d-3p [47]	Embryonic development
Oligo-Astheno-Zoospermia	Spermatozoa	Down	miR-15a, miR-15b, miR-30, miR-34b, miR-34c-5p, miR-193-5p [51, 53]	Spermatogenesis
Oligozoospermia and Teratozoospermia	Spermatozoa	Down	miR-151-5p [47]	Aging
			miR-935, miR-125a-3p [47]	Cell cycle and chromatin modification
			miR-132-5p, miR-320b, miR-195-5p [47]	Cellular component morphogenesis and cell morphogenesis

The study of miRNAs in idiopathic male infertility is complex because the genetic cause cannot be identified in many patients and due to the presence of multiple factors that can influence the phenotype. Here, we will focus on miRNAs that are up- or down-regulated in seminal plasma, spermatozoa or extracellular microvesicles (including exosomes) of infertile patients compared with controls. These results are representative of the findings reported by studies on male subfertility in the last years (Table 1). To be considered as representative, a miRNA has to be deregulated and show similar expression profiles in at least two different studies and/or by using two different techniques, such as microarray, high-throughput sequencing or RT-qPCR. In summary, six studies on male idiopathic infertility (patients with NOA, asthenozoospermia, oligozoospermia or oligoasthenozoospermia) found that five miRNAs are upregulated and four downregulated. Among these miRNAs, hsa-miR-429 [36, 51, 52] and hsa-miR-1275 [47, 53], which are upregulated, and hsa-miR-15b [47, 51, 53], which is down-regulated, are involved in spermatogenesis. Moreover, hsa-miR-141 [36, 51], which is involved in embryonic development, is upregulated. Finally, hsa-miR-19b [47, 54] and hsa-miR-483-5p [47, 53], which are upregulated, and hsa-miR-28-5p [47, 53], hsa-miR-19a [48, 53] and hsa-miR-1973 [48, 51], which are downregulated, are expressed in spermatozoa, but they have not been associated with spermatogenesis or related processes.

When focusing on the miRNA differential expression patterns in NOA, three studies pinpointed four miRNAs that are involved in spermatogenesis [36, 52, 55]: hsa-miR-34b*, hsa-miR-34c-5p and hsa-miR-122, which are downregulated [52, 55], and hsa-miR-429, which is upregulated [36, 52].

In patients with asthenozoospermia, two studies found that hsa-miR-27a [47, 56, 57], hsa-miR-548b-5p, hsa-miR-548c-5p and hsa-miR-548d-5p are up-regulated [47], while hsa-miR-34b-3p [47, 51], hsa-miR-520 h and hsa-miR-520d-3p are downregulated [47]. These miRNAs are expressed in spermatozoa and are involved in spermatogenesis (hsa-miR-34b-3p, hsa-miR-27a), embryonic development (hsa-miR-520 family) or in signaling pathways and human tumorigenesis (hsa-miR-548 family). The two last miRNA families have not yet been associated with spermatogenesis.

Comparison of the results of the two studies by Abu-Halima et al. indicates that six miRNAs are downregulated in patients with oligoasthenozoospermia compared with normozoospermic patients. Among them, hsa-miR-15a, hsa-miR-15b, hsa-miR-30, hsa-miR-34b, hsa-miR-34c-5p and hsa-miR-193b-5p have a direct role in spermatogenesis [51, 53].

Salas et al. were the first to perform miRNA profiling in spermatozoa from patients with teratozoospermia (n = 10) and oligozoospermia (n = 10) and to associate these expression patterns with semen parameters. In the teratozoospermic sperm samples, they identified eight downregulated miRNAs and eleven upregulated miRNAs. In the oligozoospermic samples, 15 miRNAs were downregulated and three were upregulated. Overall, six miRNAs were downregulated in both conditions: hsa-miR-151-5p that is related to aging; hsa-miR-935 and hsa-miR-125a-3p that are related to cell cycle and chromatin modification; hsa-miR-132-5p, hsa-miR-320b and hsa-miR-195-5p that are involved in cell and cellular component morphogenesis [47].

MicroRNAs and male infertility: molecular mechanisms

MiRNAs modulate gene expression post-transcriptionally by targeting specific mRNAs. Several studies showed that some miRNAs mediate the expression of genes that are involved in idiopathic male infertility. For instance, hsa-miR-27a is closely associated with spermatogenesis and infertility. In patients with asthenoteratozoospermia, increased expression of hsa-miR-27a mediates the repression of the Cysteine-RIch Secretory Protein2 (*CRISP2*) gene that plays a role in sperm motility, acrosome reaction and gamete fusion. Spermatozoa lacking CRISP2 exhibit low sperm motility and abnormal morphology [57].

Estrogen receptors (ERs) mediate estrogen action in the regulation of the hypothalamus-pituitary-testis axis and have a key role in male infertility. Dysfunction of this axis impairs spermatogenesis, leading to reduction of sperm density, sperm motility and morphology [58] and seminiferous tubules [59]. In sperm of patients with oligozoospermia, hsa-miR-100 and hsa-let-7b, two miRNAs that target the ER genes, are overexpressed, while ER expression levels are decreased [60]. Moreover, it has been reported that in severe oligozoospermia, hsa-miR-34c-3p downregulates Phosphatidylinositol-Specific Phospholipase C, X Domain containing 3 (*PLCXD3*). The PLCXD3 protein is expressed at later stages of spermatogenesis [61]. Therefore, these miRNAs might have a diagnostic and prognostic value in infertile men with oligozoospermia.

Very recently, Tang et al. showed that hsa-miR-210 is involved in spermatogenesis by targeting Insulin-like Growth Factor 2 (IGF2). IGF2, a component of the insulin/IGF system, regulates spermatogenesis through its generalized effects on cell proliferation, growth, differentiation and survival and also more specifically by stimulating spermatogonial DNA synthesis and Sertoli cell proliferation [62]. Therefore, hsa-miR-210 could be a potential NOA biomarker.

MicroRNAs as predictive biomarkers in ART

In ART, intra-cytoplasmic sperm injection (ICSI) is the most commonly used IVF procedure overcoming male fertility problems. Recent progress has enabled the recovery of testicular sperm from patients with NOA for ICSI. However, these spermatozoa offer a limited value for ICSI and the outcome is usually poor. Moreover, gene abnormalities, which may affect the developing zygote, are generally not assessed in the current clinical practice. Therefore, biomarkers to better evaluate semen quality are necessary. The most abundant miRNA in human sperm is hsa-miR-34c [63] and it is involved in spermatogenesis. Recently, Cui et al. showed that hsa-miR-34c levels in spermatozoa are correlated with ICSI outcomes [64]. Specifically, the percentage of good quality embryos at day 3 was higher in the group with higher hsa-miR-34c expression in spermatozoa. This finding suggests that hsa-miR-34c could become a predictive biomarker to identify defective spermatozoa.

Piwi-interacting RNAs in male infertility

Unlike miRNAs which are expressed in a variety of tissues and cells, piwi-interacting RNAs (piRNAs) are expressed mainly in germ cells especially pachytene spermatocytes and spermatids in human testes [65]. piRNAs are longer (about 29 nucleotide-long) than miRNA and form RNA-protein complexes through interactions with PIWI proteins [66]. These piRNA complexes have been linked to both epigenetic and post-transcriptional gene silencing of retrotransposons and other genetic elements in germ line cells, particularly those in spermatogenesis [67–69].

Cell-free piRNAs have been detected in human semen. Interestingly, cell-free seminal piRNAs levels gradually decrease from fertile patients to asthenozoospermia patients and then to azoospermia patients; moreover they were found to be correlated with sperm viability. A piRNA signature of 5 piRNAs (piR-31068, piR-31925, piR-43771, piR-43773 and piR-30198) that distinguishes fertile from infertile patients has been identified. This panel could be used as specific non-invasive biomarker of male infertility [12]. New studies on piRNAs should open up new avenues in research and diagnostic since they can provide firsthand information about gene disorders and dysregulation in the male reproductive system [70, 71].

Limitations in biomarker identification and validation

To identify and to validate small non-coding RNAs as infertility biomarkers, a complete survey of the literature is crucial.. Indeed, in the literature, a same miRNA can be either up- or down-regulated, leading to misinterpretations. For example, hsa-miR-122 is down-regulated in spermatozoa of patients with NOA [52] or with oligo- and asthenozoospermia [51]. On the other hand, in semen, it is downregulated in patient with NOA, but up-regulated in patients with asthenozoospermia [55]. This could reflect disorder- or tissue/cell-specific variations, but also differences in sample processing. Methodological and technical approaches are generally not uniform between laboratories and this can lead to discordant results. Moreover, as described very recently by Dong et al., misleading factors might exist when measuring seminal cf-RNA levels. For instance, heat exposure or long sexual abstinence increases semen cf-RNA levels [72]. These types of information could represent a new entry to improve the statistical analysis of raw data.

Therefore, to be considered as promising biomarker, a given small non-coding RNA should have the most significant expression difference between healthy controls and patients and between healthy and pathological tissues/cells/fluids. Moreover, it should be assessed in other relevant diseases because a candidate biomarker may have a similar expression profile also in other related disorders. Finally, validation studies should be performed using a large number of clinical samples.

Conclusions

Considering the number of studies carried out in the field, little has been clarified about circulating nucleic acids in spermatogenesis and in male infertility. Here, we provide a complete summary of the consistent data on circulating nucleic acids and intracellular miRNAs in male infertilty. Altogether, these data suggest that cf-DNAs, cf-mRNAs, miRNAs and piRNAs may be of prognostic and diagnostic value. However, a more detailed description of seminal plasma constituents is required to identify specific biomarkers of male reproductive defects. Moreover, the identification of infertility-related cf-DNAs and miRNAs in blood might provide powerful biomarkers to explore male infertility beyond semen analysis. New avenues of research could be opened for the development of new treatments of male infertility or even for the design of male contraceptive drugs.

Abbreviations

ART: Assisted reproductive technology; ATZ: Asthenoteratozoospermia; cf-DNA: Cell-free DNA; cf-RNA: Cell-free RNA; CRISP2: Cysteine-rich secretory protein2; hsa: Homo SApiens; ICSI: Intra-cytoplasmic sperm injection; IGF2: Insulin-like growth factor2; miRNA: microRNAs; mRNA: Messenger RNA; NOA: Non-obstructive azoospermia; OA: Obstructive azoospermia; piRNA: Piwi interacting RNA; PLCXD3: Phosphatidylinositol-Specific Phospholipase C, X Domain containing3; siRNA: Small interfering RNA

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AB collected data and wrote the manuscript. AG, AF, TM, SB, AP, AT and SH revised the manuscript for critical content. All authors have read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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