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480 increases tRFs in mature mouse sperm and induces insulin resistance and impaired glucose  
481 tolerance in the offspring. Moreover, the microinjection of purified tRFs from the sperm of mice  
482 raised on HFD into control zygotes replicate the metabolic phenotypes in the offspring (Chen,  
483 Yan, Cao, *et al.*, 2016). An interesting follow-up study reported that the tRNA methyltransferase  
484 DNMT2, responsible for methylating the C38 position (m<sup>5</sup>C) of tRNA<sup>Asp</sup>, tRNA<sup>Gly</sup>, and tRNA<sup>Val</sup>,  
485 is essential for sperm sncRNA-mediated transmission of HFD-induced metabolic disorders  
486 (Zhang *et al.*, 2018). Mice lacking DNMT2 showed changes in tsRNAs and rsRNA-28S content  
487 in mature spermatozoa (Zhang *et al.* 2018). The observation that HFD induces metabolic and  
488 pleasure seeking phenotypes in progeny, effects reproduced by the injection of total sperm RNA  
489 or tRF-enriched sperm RNA fraction, but not by the injection of the fraction of large sperm RNAs,  
490 corroborates this role of tRFs (Mashoodh and Ferguson-Smith, 2018). Rats exposed to HFD also  
491 exhibited alterations in piRNA, miRNA and tRF profiles (de Castro Barbosa *et al.*, 2016). Their  
492 progeny displayed reduced body weight and pancreatic beta-cell mass compared with the control  
493 group, whilst adult female offspring were glucose intolerant and resistant to HFD-induced weight  
494 gain. Even more interesting, the parents exposed to HFD and their F1 male progeny presented  
495 common DNA methylation and sncRNA expression signatures, indicating a possible  
496 intergenerational epigenetic inheritance (de Castro Barbosa *et al.*, 2016). An important caveat in  
497 considering these effects in humans is that the direct relationships between sperm quality, sperm  
498 RNAs and intergenerational responses have almost exclusively relied on and been reported in  
499 animal models. However, it has been shown that human sperm can also present a rapid response  
500 to diet, both in terms of sperm motility and tsRNA content (Nätt *et al.*, 2019). Healthy young men  
501 were exposed to a 2-step diet intervention - 1 week of healthy diet followed by 1 week of  
502 additional sugar intake - and the authors investigated three ejaculates of the same men (before the  
503 intervention, after the first week and after the second week). After the first week, sperm showed  
504 an increase in motility and, after just one week of a high-sugar diet, the sperm of these individuals  
505 revealed an upregulation of tsRNAs, especially nuclear internal T-loop tsRNA (nitRNA) (Nätt *et*  
506 *al.*, 2019). This was the first controlled study in men reporting that the sncRNA repertoire of

507 human sperm is rapidly and specifically modulated by dietary changes, despite no evidence that  
508 these changes might affect their offspring exists.

509 Although their function in sperm remains poorly explored, new data points to a possible role of  
510 tRNA<sup>Gln-TTG</sup>-derived small RNAs in regulating early cleavage events in preimplantation embryos  
511 (Chen *et al.*, 2020). Using a porcine model, Chen and colleagues demonstrated that the  
512 microinjection of antisense tRNA<sup>Gln-TTGs</sup> into IVF oocytes resulted in a diminished number of 2-  
513 cell, 4-cell and blastocyst embryos, causally attributed to an aberrant first cell cleavage (Chen *et al.*,  
514 2020). Sperm Gln-TTGs may also act by regulating cell cycle-associated genes and  
515 transposable elements (Chen *et al.*, 2020).

516 In summary, there is growing interest in tsRNAs, fuelled by evidence at least from animal models,  
517 supporting their involvement in intergenerational and transgenerational inheritance. Additionally,  
518 emerging data indicate that these fragmented tRNAs are also involved in the regulation of early  
519 embryogenesis, which should be further explored.

## 520 rRNA

521 Nuclear and mitochondrial rRNAs are transcribed as long RNA precursors subsequently spliced  
522 into functional transcripts. These are recognized as one of the most abundant RNA classes in  
523 sperm, representing approximately three-quarters of all retained RNAs (Johnson, Sendler, *et al.*,  
524 2011). Several ribosomal protein transcripts (5S, 5.8S, 18S, 28S and 45S) are differentially  
525 expressed in the sperm of asthenozoospermic and idiopathic infertile men compared with fertile  
526 patients (Bansal *et al.*, 2015). The presence of these transcripts in sperm and the abnormal profiles  
527 observed in infertile patients points to a crucial role of ribosomes in the production of high-quality  
528 sperm. However, rRNA is usually highly fragmented (rsRNA), maintaining the translational  
529 quiescent state of sperm (Jodar *et al.*, 2013; Johnson, Sendler, *et al.*, 2011). The 28S rRNA  
530 precursor-derived rsRNA (28S rsRNA) represents near 60% of all rsRNAs in mature sperm (Hua  
531 *et al.*, 2019). However, in common with tsRNA, relatively little is known of the role of rsRNA in  
532 sperm physiology.

## 533 Transposable elements

534 A large proportion of human sncRNAs are repetitive elements, mainly represented by members  
535 of the short interspersed nuclear elements (SINE)/ALU and long interspersed nuclear elements  
536 (LINE) families of transposable elements (Krawetz *et al.*, 2011). LINE1, for instance, is a  
537 transposable element with dynamic activity during mouse embryo development and cessation of  
538 its activity results in the arrest of embryonic development at the 2- or 4- cell stage (Beraldi *et al.*,  
539 2006). The possibility that transposable elements found in sperm modulate gene expression  
540 during early embryo development has not been confirmed. Thus, the function of transposable  
541 elements in the male germline and in early embryo development remains uncertain.

#### 542 Endo-siRNA

543 The siRNA is a small double-stranded RNA of ~21 nt in length. Like miRNAs, siRNA seems to  
544 act with RISC complex and argonaut proteins to target complementary RNAs, thus mediating  
545 transcriptional and post-transcriptional gene silencing (Yuan *et al.*, 2016). However, contrary to  
546 miRNAs (Figure 2), endo-siRNA production bypasses DROSHA/DGCR8 processing, being  
547 instead directly cleaved by DICER from endogenous double stranded RNA substrates (Yuan *et*  
548 *al.*, 2016).

549 A study aiming to investigate the role of miRNAs and siRNAs in fertilization and preimplantation  
550 embryonic development revealed that Dicer knockout mice have an altered set of sperm siRNAs  
551 and miRNAs; the microinjection of their sperm into wild-type (WT) oocytes resulted in embryos  
552 with reduced developmental potential (Yuan *et al.*, 2016). This subfertile phenotype can be  
553 rescued by injecting total or small RNAs derived from WT sperm into ICSI embryos (Yuan *et al.*,  
554 2016). These observations support a role for paternal miRNAs and endo-siRNAs in the control of  
555 post-transcriptional events upon fertilization and early embryo development. However, whether  
556 this rescue was due to the role of endo-siRNAs or only due to miRNAs remains unclear.

#### 557 Other sncRNAs

558 Other classes of sncRNAs present in human sperm include snRNAs, snoRNA, YRNAs and  
559 quiescent RNAs (qRNA). The presence of snRNAs in sperm was first described in 1993 in rat  
560 (Concha *et al.*, 1993). U1 and U2 snRNAs were detected in the sperm nucleus by ISH and, as

561 components of the small ribonucleoprotein particle (RNP), these may be involved in the  
562 processing of nuclear pre-mRNAs (Concha *et al.*, 1993). A minor portion of sncRNAs are  
563 YRNAs, small cytoplasmic RNAs (85-115 nt) associated with Ro protein forming an RNP  
564 complex that is involved in quality control pathways for misfolded RNAs. In human sperm,  
565 specific YRNAs fragments are possibly involved in the first stages of embryo development to  
566 initiate DNA damage repair (Krawetz *et al.*, 2011). Termed quiescent RNAs (qRNAs), >10% of  
567 sncRNAs in sperm are located in histone-enriched TSS and promoter sequences though their  
568 function remains unknown (Krawetz *et al.*, 2011).

569 A potential novel class of sncRNAs, including sperm RNAs (spR)-12 and -13 (~20 nt), identified  
570 in mouse sperm appears to be derived from additional processing of piRNAs (Kawano *et al.*,  
571 2012). These are present in one-cell embryos and maintained until the blastocyst stage, having a  
572 potential role in the maintenance of genome integrity during early embryo development (Kawano  
573 *et al.*, 2012). Additional sncRNAs include NF90-associated RNAs (snaR; ~117 nt) that are  
574 relatively abundant in human sperm (*snaR-C3*, *-C4*, *-E*, *-F*, *-G1* and *-F*) (Sendler *et al.*, 2013).  
575 *snaR-G1* levels are higher in sperm than in testes and other somatic cells and are associated with  
576 the promoter region for the beta 1 subunit of the human chorionic gonadotrophin (*hCG1*), an  
577 important hormone in implantation and embryo development (Parrott and Mathews, 2007).

### 578 **Other sperm RNAs**

579 In mature sperm, more than 65% of reads align to regions with unknown annotation or function  
580 (Sendler *et al.*, 2013). Non-coding RNAs located within the introns of coding mRNAs - intronic  
581 retained elements (IRE) - are transcribed after gene expression and seem to have a role similar to  
582 miRNAs (Hill *et al.*, 2006). In sperm, 200 different non-coding transcripts are derived from full-  
583 length introns; but the mechanisms by which these transcripts escape degradation after alternative  
584 splicing remains unclear (Sendler *et al.*, 2013). The genes in the origin of these intronic elements  
585 do not group within a specific ontological category, are not correlated with a particular expression  
586 pattern during embryo development (Vassena *et al.*, 2011), and their function remains unclear.

### 587 **Influence of sperm RNAs in pre-implantation embryo quality and embryonic development**

588 DNA damage and sperm aneuploidies have been correlated with poor fertilization and reduced  
589 rates of implantation and pregnancy. As previously described, alterations in sperm mRNA content  
590 may indicate abnormalities in spermatogenesis and lead to abnormal embryo development and  
591 growth, especially those delivered to the oocyte upon fertilization. Based on the zona-free hamster  
592 egg/human sperm penetration assays and in the comparison of sperm, eggs and embryos RNA  
593 from mouse it was found that some sperm transcripts that were not present in the unfertilized  
594 oocyte (sperm-specific transcripts) were introduced by the sperm upon fertilization (Ntostis *et al.*,  
595 2017; Ostermeier *et al.*, 2004). Furthermore, the possibility that a large subset of sperm RNAs are  
596 deeply embedded in the nuclear matrix, forming a complex with sperm DNA (Miller *et al.*, 2010),  
597 further supports this hypothesis. Most of the proteins encoded by those sperm-specific transcripts  
598 are associated with fertilization. Those include clusterin (CLU), calmegin (CLGN) and several  
599 heat shock response proteins involved in embryo development. Additionally, the injection of the  
600 sperm-borne factor I-phosphatidylinositol 4,5-biphosphate phosphodiesterase zeta (*PLCζ*)  
601 mRNA into mouse oocytes resulted in a functional calcium oscillator and egg activation,  
602 supporting its role in the control of cell cycle-dependent  $Ca^{2+}$  oscillations in early embryogenesis  
603 (Sone *et al.*, 2005). The Integrator complex subunit I (*INTSI*), which is involved in the  
604 transcription and processing of the snRNAs U1 and U2, is also retained in human sperm (Vassena  
605 *et al.*, 2011). Microarray analysis showed that the *INTSI* mRNA levels increase immediately after  
606 fertilization and before zygotic genome activation, and knockout of this gene in mouse is lethal  
607 for embryos at the blastocyst stage, supporting its potential role at the initial steps of  
608 embryogenesis (Vassena *et al.*, 2011).

609 Conversely, the evidence that noncoding RNAs appear during the final stages of spermatogenesis  
610 or are acquired during epididymal transit suggests that they may be involved in post-fertilization  
611 events (Ostermeier *et al.*, 2004). The most abundant miRNA in human sperm, miR-34c, has been  
612 described as essential for the first cellular division in mouse zygotes (Liu *et al.*, 2012). This  
613 snRNA was correlated with ICSI success, since patients with higher levels of sperm miR-34c  
614 exhibited higher numbers of good quality embryos and increased rates of implantation,  
615 pregnancy, and live births (Cui *et al.*, 2015). Recently it was reported that in bulls, sperm-borne



616 miR-216b regulates cell proliferation and the first cleavage during early embryo development,  
617 and determines blastocyst quality, specifically through *K-RAS* levels modulation (Alves *et al.*,  
618 2019). Mouse embryos generated by intracytoplasmic sperm injection (ICSI) using sperm from  
619 caput epididymis presented abnormal gene regulation likely associated with implantation failure  
620 (Conine *et al.*, 2018). Moreover, the microinjection in caput-derived embryos of miRNAs purified  
621 from cauda epididymosomes, normally gained during epididymal transit, rescued these  
622 preimplantation molecular defects and suppressed the observed post-implantation embryonic  
623 lethality (Conine *et al.*, 2018). These experimental data strongly suggests that a fraction of sperm  
624 RNAs crucial for embryogenesis is gained upon sperm entry into the epididymis. Moreover, since  
625 the sperm-mediated delivery of paternal mRNAs and small RNAs is seemingly essential for  
626 correct embryological development in mouse, evaluation of these may contribute to fertility  
627 diagnosis and the prediction of ART outcomes (Bashiri *et al.*, 2020; Conine *et al.*, 2018).  
628 Considering the apparent important role of these acquired RNAs, it is important to explain how  
629 it is possible to achieve live healthy progeny by ICSI using testicular sperm or caput epididymal  
630 sperm. In humans, the origin of the sperm used in ICSI does not appear to have a major influence  
631 on early-life outcomes for the offspring, thus questioning the genuine relevance of sperm RNAs  
632 for human implantation, embryo quality and development. Indeed, a recent meta-analysis  
633 confirmed that clinical pregnancy, live birth and abortion rates do not differ between the use of  
634 testicular sperm obtained by testicular sperm extraction (TESE) and ejaculated sperm in patients  
635 with azoospermia factor c (AZFc) microdeletions (Zhou *et al.*, 2021). Similarly, in patients with  
636 obstructive azoospermia (OA), percutaneous epididymal sperm aspiration (PESA) followed by  
637 ICSI revealed no differences in the rate of clinical pregnancy and miscarriage compared with  
638 testicular sperm aspiration (TESA) (Shih *et al.*, 2019). According to these data, we hypothesize  
639 that human testicular sperm contain sufficient amounts of those RNAs required to promote  
640 fertilization, normal development and implantation success; or that the protein levels encoded by  
641 the mRNAs or regulated by sncRNAs are sufficient to compensate for the lack of transcripts in  
642 immature sperm. Alternatively, differences between human and rodent epididymal differentiation  
643 may explain these conflicting observations (Nätt and Öst, 2020). Further research in this field



672 identification of specific paternal factors and pathways negatively affecting fertility outcomes. If  
673 sperm can provide information equivalent to invasive testicular biopsies regarding the  
674 mechanisms underlying subfertility or infertility, then a non-invasively obtained semen sample is  
675 a much more appealing and acceptable option.

#### 676 **Global Summary and final considerations**

677 The male gamete is far from being a quiescent cell, the solitary role of which is to deliver the  
678 paternal genome to the oocyte. The constant development of more sensitive and accurate RNA  
679 technologies has allowed the identification and characterization of diverse transcripts in human  
680 sperm. This complex RNA population, comprising coding and non-coding RNAs, reflects not  
681 only the past course of spermatogenesis, but may also influence fertilization, early  
682 embryogenesis, and developmental maintenance. Other predicted functions of these transcripts  
683 include paternal chromatin packaging, sperm maturation and capacitation, as well as  
684 intergenerational and transgenerational inheritance, possibly modulating the genotype and  
685 phenotype of the progeny.

686 Emergent data from animal models revealed sperm RNA content remodelling during post-  
687 testicular maturation in the epididymis, which can be modulated by environmental changes and  
688 thus, may be involved in the transmission of epigenetic information to the next generation. The  
689 epididymosomes-secreted sncRNAs seem to be crucial for fertilization and embryo development,  
690 possibly providing important diagnostic markers for some male reproductive disorders.  
691 Nevertheless, further investigation is needed to unravel the underlying mechanisms between  
692 changes in epididymal-secreted sncRNAs and male subfertility. The recognition of which specific  
693 RNAs are markers of past gene activity or have an active role in post-fertilization remains crucial.  
694 Transcriptional profiling has considerable potential for the discovery of fertility biomarkers in  
695 human medicine. The RNA fingerprint of sperm from normal fertile men could help to elucidate  
696 the underlying causes of idiopathic male fertility. Studies have emerged that attempt to define a  
697 panel of male (in)fertility biomarkers with improved discriminatory power compared with  
698 individual biomarkers (Abu-Halima *et al.*, 2014; Corral-Vazquez *et al.*, 2019; Hua *et al.*, 2019).







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**1263 Figures Legends**

1264 **Figure 1** - Origin of sperm RNA and its dynamics through the male reproductive tract. During  
1265 spermatogenesis, testicular sperm lose most of their cytoplasm and typical organelles, and  
1266 consequently, several cytoplasmic RNAs. The majority of RNAs remaining in testicular sperm  
1267 are untranslated RNAs reminiscent of spermatogenesis (~80%). After leaving the testis, sperm  
1268 are matured in the epididymis, acquiring motility during epididymal transit. Here, sperm are  
1269 exposed to epididymal fluid and extracellular vesicles (epididymosomes), losing most of their  
1270 piRNA and snoRNA content and gaining several tsRNAs. The miRNA content of epididymal  
1271 sperm is likewise strongly modulated during its journey through the different segments of the  
1272 epididymis.

1273

1274 **Figure 2** – Synthesis of miRNA, piRNA and tsRNA and their main functions. (A) miRNA is  
1275 transcribed in the nucleus from coding or non-coding genes by RNA polymerase II, as miRNA  
1276 precursors (pri-miRNA) (Khawar *et al.*, 2019). Pri-mRNAs are further processed to an  
1277 intermediate form by DROSHA (Ribonuclease 3) and DGCR8 (DiGeorge syndrome critical  
1278 region 8), transported to the cytoplasm via exportin-5' and matured by an RNase III  
1279 endonuclease (DICER) to their functional form (20-24 nt) (Khawar *et al.*, 2019). One strand of  
1280 miRNA is incorporated into an Argonaut containing ribonucleoprotein particles (RNP) leading to  
1281 the formation of a miRNA-induced silencing complex (miRISC) (Khawar *et al.*, 2019). The other  
1282 strand is usually degraded. miRNAs regulate gene expression by inhibiting or activating  
1283 translation, or by targeting mRNA for degradation, through the binding to a 3' UTR target  
1284 sequence. (B) piRNAs are usually organized in the genome as clusters ranging up to 100 kb in  
1285 size and, contrary to miRNAs, they are not associated with histones and exhibit less CpG  
1286 enrichment. They typically have uracil at the first position and result from the processing of  
1287 precursors to their mature form (24-30 nt) by a PIWI protein-dependent mechanism (Krawetz *et*  
1288 *al.*, 2011). piRNAs interact with the piwi- family proteins Piwi-like protein 1 (MIWI), Piwi-like  
1289 protein 4 (MIWI2), and Piwi-like protein 2 (MILI). Some piRNAs regulate the expression of the  
1290 associated protein-coding gene (Pantano *et al.*, 2015) and protect the genome from the adverse

1291 effects of invasive elements, such as transposons. (C) tsRNA results from the cleavage of tRNA  
1292 or pre-tRNA by endonucleases (revised by (Jin and Guo, 2019)). Two main types of tsRNAs can  
1293 be produced, according to their length and the region of tRNAs from which they are derived: (i)  
1294 tRNA-derived stress-induced RNAs (tiRNAs), that are 5'- or 3'-tRNA halves with 30-40 nt,  
1295 generated by tRNA cleavage by ribonuclease Rny1 and angiogenin near or in the anticodon loop;  
1296 and (ii) tRNA-derived fragments (tRFs), 18-22 nt sequences generated from mature tRNAs or  
1297 precursors by nucleases Dicer or RNase Z (Jin and Guo, 2019).

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**Table 1** – Summary of the types, characteristics, and roles of the most studied RNA classes in sperm.

	<b>mRNA</b>	<b>lncRNA</b>	<b>miRNA</b>	<b>piRNA</b>	<b>tsRNA</b>
<b>Length</b>	Variable	200-10,000 nt	20-24 nt	24–30 nt	18-40 nt
<b>Strand</b>	Single-stranded				
<b>Function</b>	Chromatin remodelling; <i>de novo</i> translation	Heterochromatin formation; Imprinting; DNA methylation; Transcriptional and post-transcriptional regulation	Cytosolic post-transcriptional silencing; Translational control; RNA degradation	Repression of retrotransposon; Cytosolic post-transcriptional regulation; Translational control and RNA degradation	mRNA stability, regulation, and silencing; protein binding; RNA modification, stress response
<b>Expression</b>	Expressed in all tissues	Expressed in all tissues	Expressed in most tissues	Mainly expressed in germ cells (spermatocytes, spermatids)	Highly expressed in sperm
<b>Main location in sperm</b>	Head, midpiece and tail	Head (nuclear or peri-nuclear compartment)	Head (Nuclear or peri-nuclear compartment); tail	Tail	Head
<b>Function in sperm</b>	Fertilization; delivery of sperm RNAs to the oocyte/ early embryo development	Transgenerational inheritance; gene expression regulation/histone modification; early embryo development	Transcriptional gene silencing; early embryonic histone replacement; early embryogenesis; epigenetic modification; sperm maturation	Protection from transposons; genome recognition and consolidation-confrontation; epigenetic modification	Epigenetic modification and transgenerational inheritance, early embryo development

**Table 2** – Studies investigating sperm RNA as biomarkers of men’s fertility and health and RNAs differentially expressed in each condition studied.

Study	Condition	RNA type	Method	Differentially expressed RNAs
(Jedrzejczak <i>et al.</i> , 2006)	Infertile men	mRNA	RT-qPCR	<i>CCR5</i> and <i>P450arom</i> mRNA (down)
(Avendano <i>et al.</i> , 2009)	Infertile men	mRNA	RT-qPCR	<i>PSG1</i> and <i>HLA-E</i> mRNA (down)
(Garrido <i>et al.</i> , 2009)	Idiopathic Infertile men	mRNA	Microarray RT-qPCR	<i>TRY1</i> , <i>GGT1</i> and <i>CAB39L</i> mRNA (down)
(Depa-Martynow <i>et al.</i> , 2012)	Idiopathic or mild male factor infertile men	mRNA	RT-qPCR	<i>PRM1/PMR2</i> mRNA (down)
(Rogenhofer <i>et al.</i> , 2013)	Subfertile men	mRNA	RT-qPCR	<i>PRM1/PRM2</i> mRNA (down)
(Bansal <i>et al.</i> , 2015)	Idiopathic infertile and Asthenozoospermic male	mRNA and lincRNAs	Microarray	<u>Asthenozoospermic</u> : <i>RPL24</i> , <i>RPL4</i> , <i>RPL9</i> , <i>RPL18</i> , <i>RPL11</i> , <i>RPL28</i> , <i>RPL35</i> and <i>RPS16</i> , <i>CANX</i> , <i>NONO</i> , <i>RHOA</i> , <i>OAZ1</i> , <i>FAU</i> , <i>SLC25A3</i> , <i>HNRNPM</i> , <i>C1D</i> , <i>PRPF8</i> , <i>HTN3</i> , <i>CERCAM</i> , <i>GDI2</i> , <i>PARK7</i> (up); <i>RPS13</i> , <i>RPL27</i> , <i>RPS24</i> , <i>RPS11</i> , <i>RPS5</i> , <i>RPS27A</i> , <i>RPL30</i> , <i>RPL34</i> , <i>RPS25</i> , <i>DAD1</i> , <i>ILF2</i> , <i>SRSF9</i> , <i>HSP90AB1</i> , <i>EIF4G2</i> , <i>HNRNPC</i> , <i>SMARCAD1</i> , <i>HINT1</i> , <i>KIFAP3</i> (down) <u>Idiopathic infertility</u> : <i>RPS25</i> , <i>RPS11</i> , <i>RPS13</i> , <i>RPL30</i> , <i>RPL34</i> , <i>RPL27</i> , <i>RPS5</i> , <i>HINT1</i> , <i>HSP90AB1</i> , <i>SRSF9</i> , <i>EIF4G2</i> , <i>ILF2</i> , <i>RPL9</i> , <i>OAZ1</i> , <i>RPL18</i> , <i>RPL35</i> , <i>FAU</i> , <i>CAPNS1</i> , <i>FAM153C</i> , <i>ARF1</i> , <i>CFL1</i> , <i>RPL19</i> , <i>USP22</i> (up); <i>DAD1</i> , <i>ZNF90</i> , <i>SMNDC1</i> , <i>c14orf126</i> , <i>HNRNPK</i> (down)
(Hamad, 2019)	Infertile couples	mRNA	RT-qPCR	<i>PRM1</i> , <i>PMR2</i> , <i>H2A</i> , <i>H2B</i> mRNA (down)
(Hazem <i>et al.</i> , 2020)	Infertile men	mRNA	RT-qPCR	<i>CBR1</i> and <i>CBR2</i> mRNA (down)
(Liu <i>et al.</i> , 2010)	Asthenozoospermic men	mRNA	RT-qPCR	<i>VDAC2</i> mRNA (up)
(Jodar <i>et al.</i> , 2012)	Asthenozoospermic men	mRNA	Microarray RT-qPCR	<i>HNRPA3</i> , <i>MT-ND2</i> , <i>ANXA2</i> , <i>LOC100289246</i> , <i>FLJ45445</i> , <i>ANKRD9</i> , <i>OAZ3</i> , <i>C1orf148</i> , <i>FLJ45340</i> , <i>C7orf30</i> , <i>MT-ND3</i> , <i>LOC100132147</i> ,

				<i>GABRB1</i> , <i>TBC1D3</i> , <i>LOC474358</i> , <i>BRD2</i> , <i>PRM1</i> and <i>PRM2</i> (down); <i>FLJ32605</i> , <i>DKFZP434B061</i> (up) <b>Validated by PCR:</b> <i>ANXA2</i> , <i>BRD2</i> and <i>OAZ3</i> mRNA (up)
(Chen <i>et al.</i> , 2012)	Asthenozoospermic and oligoasthenozoospermic men	mRNA	RT-qPCR	<i>NRF2</i> mRNA (down)
(Pelloni <i>et al.</i> , 2018)	Asthenozoospermic men	mRNA	RT-qPCR	<i>ROPNI</i> and <i>CABYR</i> mRNA (down)
(Caballero-Campo <i>et al.</i> , 2020)	Asthenozoospermic high (A-F1) and low (A-F2) motile fraction vs normozoospermic high (N-F1) and low (N-F2) motile fraction	mRNA	Microarray	A-F1 vs N-F1: 116 up- and 1747 down-regulated genes A-F2 vs N-F2: 17 up- and 190 down-regulated genes N-F1 vs N-F2: 7 up- and 54 down-regulated genes A-F1 vs A-F2: 42 up- and 866 down-regulated genes
(Zheng <i>et al.</i> , 2011)	Oligoasthenozoospermic men	mRNA	RT-qPCR	<i>BDNF</i> mRNA (down)
<b>(Steger <i>et al.</i>, 2008)</b>	Oligozoospermic men	mRNA	RT-qPCR	<i>PRM1</i> mRNA (down) and <i>Bcl2</i> mRNA (up)
(Montjean <i>et al.</i> , 2012)	Oligozoospermic men	mRNA	Microarray RT-qPCR	157 transcripts up- or down-regulated. <b>Validated by PCR:</b> <i>TPD52L3</i> , <i>PRM2</i> , <i>JMJD1A</i> and <i>NIPBL</i> (down)
(Savadi-Shiraz <i>et al.</i> , 2015)	Teratozoospermic men	mRNA	RT-qPCR	<i>PRM1</i> and <i>PRM2</i> (down); <i>TNP2</i> (up)
(Giebler <i>et al.</i> , 2018)	Normozoospermic vs Non-normozoospermic men	mRNA	RT-qPCR	<i>PIWI-LIKE 1</i> (up) and 2 (down) mRNA
(Ferlin <i>et al.</i> , 2010)	Varicocele patients	mRNA	RT-qPCR	<u>Varicocele and oligozoospermia:</u> <i>HSPA4</i> , <i>HSF1</i> , <i>HSF2</i> mRNA (up); <u>Varicocele:</u> <i>HSFY</i> (up)
(Linschooten <i>et al.</i> , 2009)	Smokers	mRNA	Microarray	781 genes differentially expressed
(Depa-Martynów <i>et al.</i> , 2007)	Patients in which IVF failed	mRNA	RT-qPCR	<i>Fertilin β</i> , <i>PRM1</i> , <i>PRM2</i> (down)

(García-Herrero, Meseguer, <i>et al.</i> , 2010)	Sperm samples with which pregnancy was not achieved with IUI (NP)	mRNA	Microarray	741 transcripts absent and 976 transcripts exclusively expressed in group NP
<b>(García-Herrero, Garrido, <i>et al.</i>, 2010)</b>	Sperm samples with which pregnancy was not achieved with ICSI (NP)	mRNA	Microarray RT-qPCR	44 sequences downregulated and 5 upregulated in group NP <b>Validated by PCR:</b> <i>IFI30</i> , <i>S100A6</i> , <i>CTSZ</i> (down) and <i>COX7B2</i> (up)
(Bonache <i>et al.</i> , 2012)	Normozoospermic donors used for therapeutic IUI vs Normozoospermic general donors	mRNA	TaqMan Arrays RT-qPCR	<i>EIF5A</i> , <i>RPL13</i> , <i>RPL23A</i> , <i>RPS27A</i> , <i>RPS3</i> , <i>RPS8</i> and <i>TOMM7</i> mRNA <b>Validated by PCR:</b> <i>EIF5A</i> , <i>RPL13</i> , <i>RPL23A</i> and <i>RPS27A</i> mRNA
(Marczylo <i>et al.</i> , 2012)	Smokers vs Non-smokers	miRNA	Microarray RT-qPCR	hsa-mir-365, hsa-mir-944, hsa-mir-1267, hsa-mir-340, hsa-mir-4513, hsa-let-7a-2-3p, hsa-mir-576-3p, hsa-mir-576-5p, hsa-mir-1246, hsa-mir-30c, hsa-mir-933, hsa-mir-7, hsa-mir-1285, hsa-mir-1270, hsa-mir-509-5p, hsa-mir-146b-3p, hsa-mir-3145-3p, hsa-mir-4748, hsa-mir-519d, hsa-mir-550a, hsa-mir-550b (up); hsa-mir-574-5p, hsa-mir-3145-5p, hsa-mir-146b-5p, hsa-mir-634, hsa-mir-129-3p, hsa-mir-652, hsa-mir-4723-5p (down) <b>Validated by PCR:</b> miR-340, miR-365 (up); miR-129-3p and miR-634 (down)
(Metzler-Guillemain <i>et al.</i> , 2015)	Smokers vs Non-smokers	miRNA	Microarray RT-qPCR	5 mRNA and 16 miRNAs upregulated, and 10 mRNAs and 7 miRNAs downregulated. <b>Validated by PCR:</b> hsa-miR-296-5p, hsa-miR-3940 and hsa-miR-520d-3p (down)
(Li <i>et al.</i> , 2012)	Men living in an environment contaminated with electronic waste vs Men living in a normal environment	miRNA	Microarray	73 significantly upregulated and 109 downregulated miRNAs <b>Validated by PCR:</b> hsa-miR-208a, hsa-miR-155, hsa-miR-222, hsa-miR-183, hsa-miR-205, hsa-miR-106a, hsa-miR-33b, hsa-miR-223, hsa-miR-10b (up); hsa-miR-363, hsa-let-7d (down)

(Abu-Halima <i>et al.</i> , 2013)	Asthenozoospermic and oligoasthenozoospermic men	miRNA	Microarray RT-qPCR	<u>Asthenozoospermic</u> : 50 miRNAs upregulated, and 27 miRNAs downregulated <u>Oligoasthenozoospermic</u> : 42 miRNAs upregulated, and 44 miRNAs downregulated <b>Validated by PCR</b> : miR-34b, miR-122 and miR-1973 (asthenozoospermic); miR-34b, miR-34b*, miR-15b, miR-34c-5p, miR-122, miR-449a, miR-1973, miR-16, and miR-19a (oligoasthenozoospermic)
(Abu-Halima <i>et al.</i> , 2014)	Subfertile, Nonobstructive azoospermia [NOA] patients	miRNA	RT-qPCR	hsa-miR-34b, hsa-miR-34b, hsa-miR-34c-5p and hsa-miR-122 (down); hsa-miR-429 (up)
(Said <i>et al.</i> , 2014)	Asthenozoospermic, teratozoospermia and infertile male	mRNA	RT-qPCR	P450arom mRNA (down)
(Salas-Huetos <i>et al.</i> , 2016)	Infertile men	miRNA	RT-qPCR	45 up- and 12 down-expressed miRNAs: hsa-miR-518f-3p, hsa-miR-208a, hsa-miR-34a-5p, hsa-miR-636, hsa-miR-520d-3p, hsa-miR-708-5p, hsa-miR-483-5p, hsa-miR-212-3p, hsa-miR-1254, hsa-miR-324-3p, hsa-miR-491-5p, hsa-miR-564 (down); hsa-miR-149-3p, hsa-miR-596, hsa-miR-346, hsa-miR-1296, hsa-miR-1298, hsa-miR-30d-3p, hsa-miR-935, hsa-miR-766-3p, hsa-miR-659-3p, hsa-let-7a-5p, hsa-miR-103a-3p, hsa-miR-193b-5p, hsa-miR-339-5p, hsa-miR-365a-3p, hsa-miR-942, hsa-miR-22-3p, hsa-let-7f-5p, hsa-miR-130b-5p, hsa-miR-644a, hsa-miR-432-3p, hsa-miR-552, hsa-miR-487a, hsa-miR-526b-5p, hsa-miR-744-5p, hsa-miR-145-5p, hsa-miR-543, hsa-miR-222-5p, hsa-miR-517-5p, hsa-miR-340-3p, hsa-miR-296-5p, hsa-miR-518d-5p, hsa-miR-520, hsa-miR-93-3p, hsa-miR-132-5p, hsa-miR-520c-3p, hsa-miR-622, hsa-miR-573, hsa-miR-

				9-3p, hsa-miR-181a-2-3p, hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-28-5p, hsa-miR-331-3p, hsa-miR-15b-5p, hsa-miR-183-3p (up)
(Salas-Huetos <i>et al.</i> , 2015)	Infertile (asthenozoospermic, teratozoospermic, oligozoospermic) men	miRNA	TaqMan miRNA array	32 differentially expressed miRNAs in the asthenozoospermic group (26 up- and 6 down-regulated), 19 in the teratozoospermic group (11 up- and 8 down-regulated), and 18 in the oligozoospermic group (3 up-regulated and 15 down-regulated) in relation to fertile control group; <b>Validated by PCR:</b> hsa-miR-34b-3p (age); hsa-miR-629-3p (motility); hsa-miR-335-5p, hsa-miR-885-5p, and hsa-miR-152-3p (concentration)
(Muñoz <i>et al.</i> , 2015)	Oligozoospermic men	miRNA	RT-qPCR	miR-449a, miR-34c-5p, miR-34b and miR-122 (down) <b>Validated by PCR:</b> miR-34b and miR-122
(Zhu <i>et al.</i> , 2019)	Oligospermic men	miRNA	RT-qPCR	miR-122-5p (down)
(Wenhao <i>et al.</i> , 2015)	Oligoasthenozoospermic men	miRNA	Microarray	32 miRNAs showed significant differences in expression between the OA and NC groups <b>Validated by PCR:</b> Hsa-miR-15a Hsa-miR-19b Hsa-miR-21 Hsa-miR-23a Hsa-miR-24 Hsa-miR-27a, Hsa-miR-142-5p (up); Hsa-miR-132, Hsa-miR-449a Hsa-miR-552 (down)
(Zhou <i>et al.</i> , 2016)	Asthenozoospermic men	miRNA	RT-qPCR	miR-27a (up)
(Heidary <i>et al.</i> , 2019)	Asthenozoospermic men	miRNA	RNA-seq	miR-206, miR-1-3p, miR-888-3p, miR-1299 (up); miR-625-3p, miR-7153-5p, miR-328-3p, miR-92b-3p, miR-27a-5p, miR-143-5p, miR-194-3p, miR-1273g-3p, miR-197-3p, miR-296-5p, miR-6510-3p, miR-3609, miR-4326, miR-26b-3p (down) <b>Validated by PCR:</b> miR-888-3p (up)
(Heidary <i>et al.</i> , 2020)	Asthenozoospermic vs men	miRNA	RT-qPCR	miR-4485-3p (down)



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(Cui <i>et al.</i> , 2018)	Patients with idiopathic male infertility who had undergone first ICSI vs Fertile patients	piRNA	RT-qPCR	hsa-piR-31704, hsa-piR-39888 (down)
(Zhang <i>et al.</i> , 2015)	Asthenozoospermic vs oligoasthenozoospermic men	lncRNA HOTAIR	RT-qPCR	HOTAIR (down)

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**Table 3** – Studies with significance for the involvement of sperm small RNAs in paternal intergenerational and transgenerational transmission.

Stimuli studied	Organism	Method	Differentially expressed sncRNAs	Microinjection	Altered offspring phenotype and epigenome	Ref.
<b>Chronic stress</b>	Mouse	RNA-seq, RT-qPCR	UP: miRNAs miR-375-3p, miR-375-5p, miR-200b-3p, miR-672-5p and miR-466-5p DOWN: piRNA cluster 110	Yes	Depressive behaviour and metabolic alterations	(Gapp <i>et al.</i> , 2014)
<b>Early life stress</b>	Mouse and human	Microarray; RT-qPCR	DOWN: miR-449a, miR-449b, miR-34b and miR-34c	No	Decrease in miR-449a and miR-34c levels in embryos at the 2-cell, 4-cell, 8-cell, and in the sperm of adult offspring from stressed fathers, consistent with findings that these mice transmit stress phenotypes to their F2 offspring	(Dickson <i>et al.</i> , 2018)
<b>Chronic ethanol consumption</b>	Mouse	RNA-seq, RT-qPCR	UP: tDR Glu-CTC, tDR His-GTG, miR-10a, miR-99b DOWN: tDR Ser-AGA, tDR Pro-AGG	No		(Rompala <i>et al.</i> , 2018)
<b>Salient olfactory experience</b>	Mouse	RNA-seq	miRNA targeting processes like regulation of cell growth, cellular response to chemical and amino acid stimulus (not specified)	Yes	Behavioural sensitivity to odour A and increased representation of Odour A-related neuroanatomy in the adult olfactory system	(Aoued <i>et al.</i> , 2020)
<b>Infection by <i>Toxoplasma gondii</i></b>	Mouse	RNA-seq	UP: miRNA count; 75 miRNAs DOWN: piRNA count; 35 miRNAs	Yes	Sexually dimorphic changes in the anxiety-like phenotype, learning, and memory in the F1 as well as F2 generations	(Tyebji <i>et al.</i> , 2020)
<b>Physical health</b>	Human	RNA-seq	UP: piR-has-11690; piR-26632 DOWN: miR-3653-3p; piR-28160; piR-27503; piR-17444	No		(Ingerslev <i>et al.</i> , 2018)

<b>Diet</b>						(Fullston
<i>High fat</i>	Mouse	Microarray, RT-qPCR	UP: miRNA-133b-3p, miRNA-196a-5p, miRNA-205-5p; DOWN: miRNA-340-5p	No	Increased body weight in females, impaired glucose tolerance and insulin resistance	<i>et al.</i> , 2013)
<i>Western-like</i>	Mouse	RNA-seq, RT-qPCR	UP: miRNAs and piRNAs miRNA-19b DOWN: miRNAs and piRNAs	Yes	Increased body weight, impaired glucose tolerance	(Grandjean <i>et al.</i> , 2015)
<i>High fat</i>	Mouse	RNA-seq, MS, PCR	UP: miRNAs and tRF m5C and m2G DOWN: miRNAs and tRFs	Yes	Impaired glucose tolerance	(Chen, Yan, and Duan, 2016; Chen, Yan, Cao, <i>et al.</i> , 2016)
<i>Low protein</i>	Mouse	RNA-seq, RT-qPCR	UP: miRNAs, piR-NAs and tRF-Gly-CCC, - TCC, -GCC, tRF-Lys-CTT, tRF-His-GTG DOWN: piRNAs, tRFs and miRNA-let7 family	Yes	Decreased expression of gene targets regulated by the endogenous retroelement MERVL in two- cell embryos. Low Protein RNAs could inhibit tRF-Gly-GCC targets in 2-cell embryos.	(Sharma <i>et</i> <i>al.</i> , 2016)
<i>High fat</i>	Rat	RNS-seq and qPCR	UP: let7c-5p, piRNA-025883, piRNA- 015935, tRF-Glu-CTC, -TTC DOWN: miRNA-293-5p, miRNA-880-3p, piRNA-036085	No	Decreased body weight, impaired glucose tolerance in females	(de Castro Barbosa <i>et</i> <i>al.</i> , 2016)
<i>High fat</i>	Mouse	RNA-seq, qPCR	UP: tsRNAs; DOWN: rsRNA-28S	Yes	Glucose intolerance	(Zhang <i>et</i> <i>al.</i> , 2018)

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<i>High sugar</i>	Human	RNA-seq, qPCR	UP: tsRNA; DOWN: rsRNA	No	(Nätt <i>et al.</i> , 2019)
<b><i>Obesity</i></b>	Human	RNA-seq	tRF; piRNA; miRNA; snRNA	No	(Donkin <i>et al.</i> , 2016)

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For Peer Review

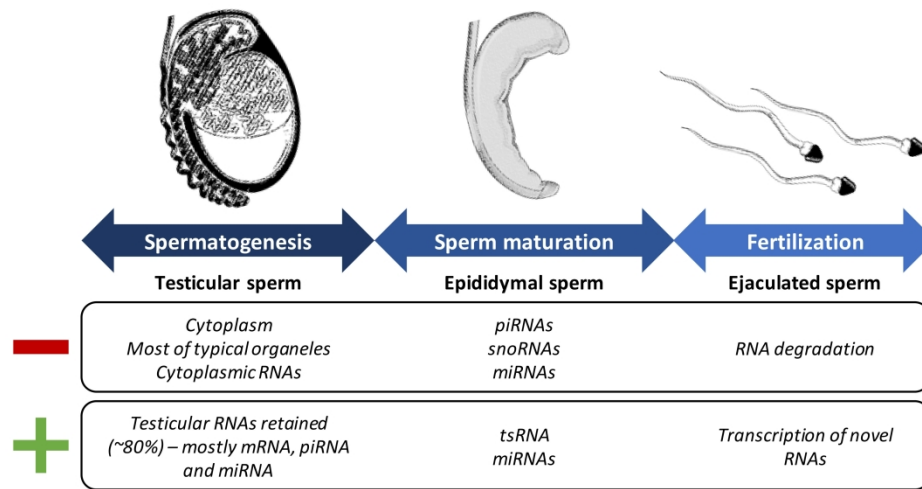


Figure 1 - Origin of sperm RNA and its dynamics through the male reproductive tract. During spermatogenesis, testicular sperm lose most of their cytoplasm and typical organelles, and consequently, several cytoplasmic RNAs. The majority of RNAs remaining in testicular sperm are untranslated RNAs reminiscent of spermatogenesis (~80%). After leaving the testis, sperm are matured in the epididymis, acquiring motility during epididymal transit. Here, sperm are exposed to epididymal fluid and extracellular vesicles (epididymosomes), losing most of their piRNA and snoRNA content and gaining several tsRNAs. The miRNA content of epididymal sperm is likewise strongly modulated during its journey through the different segments of the epididymis.

1124x625mm (96 x 96 DPI)

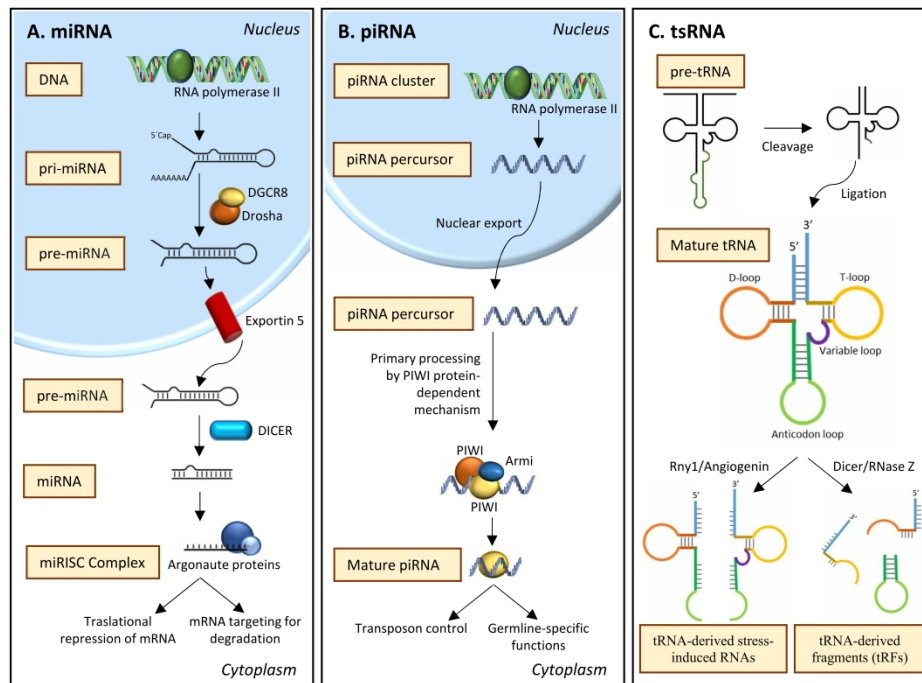


Figure 2 – Synthesis of miRNA, piRNA and tsRNA and their main functions. (A) miRNA is transcribed in the nucleus from coding or non-coding genes by RNA polymerase II, as miRNA precursors (pri-miRNA) (Khawar et al., 2019). Pri-mRNAs are further processed to an intermediate form by DROSHA (Ribonuclease 3) and DGCR8 (DiGeorge syndrome critical region 8), transported to the cytoplasm via exportin-5' and matured by an RNase III endonuclease (DICER) to their functional form (20-24 nt) (Khawar et al., 2019). One strand of miRNA is incorporated into an Argonaute containing ribonucleoprotein particles (RNP) leading to the formation of a miRNA-induced silencing complex (miRISC) (Khawar et al., 2019). The other strand is usually degraded. miRNAs regulate gene expression by inhibiting or activating translation, or by targeting mRNA for degradation, through the binding to a 3' UTR target sequence. (B) piRNAs are usually organized in the genome as clusters ranging up to 100 kb in size and, contrary to miRNAs, they are not associated with histones and exhibit less CpG enrichment. They typically have uracil at the first position and result from the processing of precursors to their mature form (24-30 nt) by a PIWI protein-dependent mechanism (Krawetz et al., 2011). piRNAs interact with the piwi- family proteins Piwi-like protein 1 (MIWI), Piwi-like protein 4 (MIWI2), and Piwi-like protein 2 (MILI). Some piRNAs regulate the expression of the associated protein-coding gene (Pantano et al., 2015) and protect the genome from the adverse effects of invasive elements, such as transposons. (C) tsRNA results from the cleavage of tRNA or pre-tRNA by endonucleases (revised by (Jin and Guo, 2019)). Two main types of tsRNAs can be produced, according to their length and the region of tRNAs from which they are derived: (i) tRNA-derived stress-induced RNAs (tiRNAs), that are 5'- or 3'-tRNA halves with 30-40 nt, generated by tRNA cleavage by ribonuclease Rny1 and angiogenin near or in the anticodon loop; and (ii) tRNA-derived fragments (tRFs), 18-22 nt sequences generated from mature tRNAs or precursors by nucleases Dicer or RNase Z (Jin and Guo, 2019).

1856x1312mm (96 x 96 DPI)