Sperm gene expression profile is related to insemination pregnancy rate and predictive of low fecundity in normozoospermic men

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Complete List of Authors:	Bonache, Sandra; IDIBELL, Human Molecular Genetics Group Mata, Anna; Fundació Puigvert, Andrology Service, Laboratory of Seminology and Embryology Ramos, María Dolores; IDIBELL, Molecular Genetic Diagnostic Center Bassas, Lluís; Fundació Puigvert, Andrology Service, Laboratory of Seminology and Embryology Larriba, Sara; IDIBELL, Human Molecular Genetics Group
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SPERM GENE EXPRESSION PROFILE IS RELATED TO INSEMINATION PREGNANCY RATE AND PREDICTIVE OF LOW FECUNDITY IN NORMOZOOSPERMIC MEN

Running title Gene expression profile and fertilizing quality of sperm

Sandra Bonache[a#], Ana Mata[b], María Dolores Ramos[c], Lluís Bassas[b*], and Sara Larriba[a]*

[a.] Human Molecular Genetics Group-[Bellvitge Biomedical Research Institute-] IDIBELL, L'Hospitalet de Llobregat, 08908 Barcelona, Spain [b.] Laboratory of Seminology and Embryology, Andrology Service-Fundació Puigvert, 08025 Barcelona, Spain [c.] Molecular Genetic Diagnostic Center- IDIBELL, L'Hospitalet de Llobregat, 08907 Barcelona, Spain

Current address: Oncogenetics Laboratory, University Hospital Vall d'Hebron, 08035 Barcelona, Spain

***Correspondence address:**

Tel: +34 932 60 74 25 (ext. 7338); FAX:+34 932 60 74 14; E-mail: slarriba@idibell.cat

Tel: +34 934 16 97 00 (ext 4440); E-mail: lbassas@fundacio-puigvert.es

ABSTRACT

Background Assessment of male fertility is traditionally based on microscopic evaluation of semen. However, the classical semen parameters do not adequately reflect the sperm function and their clinical value in predicting fertility is limited. We hypothesize that sperm expression profile could reflect the fertilizing quality of spermatozoa and could be more informative to predict the *in vivo* reproductive fitness of men with normal semen parameters. **Methods** Sperm gene expression patterns of 68 normozoospermic donors (43 phase I and 25 phase II), used for therapeutic intrauterine insemination (IUI), were analysed via TaqMan Arrays. **Results** Significant differences in expression of individual genes were observed between groups of donors with the lowest and highest pregnancy rates after IUI. Additionally, we have developed a molecular means to classify the fertility status of semen donors for IUI based on the expression signature of 4 genes. In the phase I study this model had 90% sensitivity and 97% specificity for discriminating donors resulting in low pregnancy rates (cut-off value: <13.6%), far better than that obtained from the combination of sperm parameters. The translation of the model was validated in phase II donors resulting in a sensitivity of 71.5% and a specificity of 78%. **Conclusions** Our findings contribute to the search for the most valuable genetic markers potentially useful as tools for predicting the pregnancy outcome. Our expression model could be a complement of classical semen analysis in order to identify sperm donors with a less favourable IUI reproductive outcome despite having normal semen parameters; it could also have a role in the study of sperm function in couples with unexplained infertility.

Key words: spermatozoa, gene expression profiling, therapeutic sperm insemination, male fertility, male infertility.

INTRODUCTION

Assessment of male fertility is based on the descriptive information provided by the basic semen analysis including: sperm count, motility and morphology. New threshold values for semen parameters have been recently updated (Cooper et al., 2010) using men who had produced a recent pregnancy as reference individuals. However, despite a clear correlation between semen quality and the probability of conception (Guzick et al., 2001), the wide overlap of measurements between fertile and infertile men suggest that semen analysis has a limited power to predict fecundity and to diagnose male infertility (Bartoov et al., 1993). The significant proportion of couples with unexplained infertility suggests that abnormal sperm function can be due to molecular defects in some cases (Lewis, 2007). Many efforts have been made to build up new diagnostic tests to provide more accurate information on the fertilizing potential of human spermatozoa (Samplaski et al., 2010) but none of them have yet met the requirements so as to be adopted for clinical purposes.

Spermatozoa contain, besides the haploid genetic material, an abundant number of functionally viable transcripts (Krawetz, 2005; Ostermeier et al., 2002; Zhao et al., 2006), commonly considered as remnants of stored mRNA from post-meiotically active genes reflecting the accurate development of spermatogenesis (review in (Miller and Ostermeier, 2006)). However, the potential for an active post-meiotic production of transcripts exists: a persistence of a low but detectable level of transcription and translation in mature sperm cells had been described (Miteva et al., 1995; Gur and Breitbart, 2006; Naz, 1998). Furthermore, human spermatozoa can deliver mRNA to the oocyte during fertilization (Ostermeier et al., 2004). Some of these mRNAs have been shown to be translated *de novo* in the oocyte after fertilization supporting the hypothesis that at least some transcripts might have a function during or beyond the process of

fertilization (Gur and Breitbart, 2006; Braude et al., 1988; Siffroi and Dadoune, 2001) and also contribute to the early transcriptome of the embryo (Boerke et al., 2007).

It has been suggested that sperm mRNAs present in the ejaculated spermatozoa represent a genetic fingerprint, and could be considered to be a historical record of what happened in gene expression during spermatogenesis (Zhao et al., 2006). Some studies have reported differences in the amount of certain sperm transcripts between infertile and fertile men (Steger et al., 2008; Avendano et al., 2009). A different expression signature was also determined related to the differences of sperm concentration (Guo et al., 2007), motility (Carreau et al., 2007) and morphology (Platts et al., 2007). Interestingly, differences in expression of a few hundreds of transcripts between fertile and infertile men with normal semen parameters have been described recently (Garrido et al., 2009). However, there is no formal study that assesses the diagnostic efficiency of sperm RNA expression in comparison to classic semen parameters.

Assisted reproduction techniques (ART) have revolutionized the treatment of infertile couples. Among them, therapeutic donor insemination (TDI) of sperm provides an ideal first approach to achieve pregnancy in couples with a severe male infertility factor. Despite having apparently normal semen characteristics, some sperm donors have low pregnancy rates (PR) after TDI (Johnston et al., 1994; Marshburn et al., 1992), a situation analogous to the male partner in couples with unexplained infertility. We hypothesize that assessment of sperm gene expression profile could reflect the fertilizing quality of spermatozoa and could also be informative in predicting *in vivo* reproductive fitness of men with normal semen parameters. To address this issue we have studied a cohort of semen donors with good semen quality and with a detailed record of reproductive outcome using intrauterine insemination (IUI) in different female recipients. Recruitment of semen donors was carried out among young university

students with unknown fertility status at the time of donation, so they were representative of the normozoospermic general population. We believe that this approach is suitable for investigating the molecular features of unexplained male infertility, because it circumvents some of the shortcomings present when studying infertile couples, such as the confounding role of the significant proportion of female causes that contribute to reproductive failure.

MATERIALS AND METHODS

Experimental design

The study was divided into two phases. In phase I, or the training phase, a general overview of gene expression behaviour was determined in relation to the PR obtained by sperm donors and a gene set expression signature was obtained. In phase II, we validated the gene set signature as a predictive diagnostic tool in an independent series of donor semen samples. The study was approved by the Institutional Review Board of the Centre.

Selection of semen donors

Recruitment of candidate donors was carried out among university students, most of whom had not attempted procreation at the time of assessment. The clinical procedures for screening semen donors were done at the Andrology Service of the Fundació Puigvert, and included full personal and familiar medical history to rule out heritable conditions, physical examination and a minimum of two semen analyses [performed in accordance with the World Health Organization guidelines (WHO, 1999) except for motility assessments, that were done at room temperature]. Spermiograms included volume, pH, sperm concentration, four-category motility assessment, vitality, morphology and antisperm antibodies. Motility and sperm count were done in duplicate aliquots of ≥200 cells, and measures were adopted to control for acceptable differences between duplicates. Sperm concentration was performed on diluted, immobilized samples using haemocytometer chambers. Computer assisted sperm analysis (CASA) was performed on fresh ejaculates with a Hamilton-Thorn 2030 system (software version 6.4) to obtain objective measurements of sperm kinematics (Pedigo et al., 1989). Serological tests for HIV I and II, hepatitis B and C, cytomegalovirus and

syphilis were done at baseline, at the end of the donations and again after six months of quarantine; only donors that tested negative were used. Karyotype analysis was done in donors enrolled after the year 2000. Donors were allowed to give 6 to 12 donations. Semen parameters of each individual donation were measured to monitor semen quality relative to baseline assessment. Donors with deteriorating semen quality were discontinued before completing the donations.

All semen samples were frozen within two hours of collection in an equal volume of glycerol-egg-yolk-citrate cryopreservative medium (Sperm Freezing medium, Irvine Scientific, Santa Ana, CA, USA) in vapours of liquid nitrogen using 1.8 mL cryovials, and stored at -196°C until needed. Cryosurvival was assessed as the percent progressive motility of sperm after thawing in a 37ºC bath.

Our study recruited a total of 68 normozoospermic donors. The inclusion criteria were as follows: a) having at least 4 surplus frozen aliquots (0.5 mL straws) available after the use for insemination purposes, b) average sperm concentration ≥40 millions/mL; progressive motility ≥ 30%, normal morphology ≥7% at the time of initial assessment, c) >10 insemination cycles per sperm donor performed to a minimum of 6 female recipients and d) not being discontinued due to impairment of semen quality during their donation period. All donors were of Caucasian origin.

Approximately 2/3 of donors (n=43) were randomly chosen for the training phase I gene expression analysis. Semen samples from the rest of individuals (n=25) were used for the validating phase II (Fig. 1).

Female recipients and insemination procedures

Women entering the TDI program at the Fundació Puigvert who were inseminated with samples from the selected donors during the period 1994 to 2006 were considered for

this study. Eligibility for insemination included severe male factor in the majority of cases, and ejaculatory disturbances or hereditary conditions in the husband. Ovulatory status was studied by biphasic temperature charts and progesterone at midluteal phase, and a normal hysterosalpingography was required before inseminations.

Previous to IUI, in all cases mild follicular stimulation was induced with 75 UI/day of gonadotrophins (Neo-Fertinorm or Pergonal, Serono SA, Spain), and monitored by analysis of estradiol and transvaginal ultrasonography. Ovulation was induced by 10000 UI of HCG (Profasi, HCG Lepori) when at least one follicle of >18 mm was observed. Thawed semen samples (0.5 mL) were diluted with 2 mL of Ham's F-10 medium with 0.5% HSA and prepared by differential centrifugation using 90 and 65% density gradients (Percoll, Pharmacia, Sweden, or Puregon, Vitrolife, Denmark) as described elsewhere (Ruiz-Romero et al., 1995). Final volume was adjusted to 0.4 mL. Inseminations of sperm were done on two consecutive days, 24 and 48 hours after the administration of HCG using an insemination catheter (#4220, Gynétics Medical, Lommel, Belgium). If β-HCG levels were increased 2 to 4 weeks after the inseminations, pregnancy was confirmed by ultrasound scan. Selection of semen donors for insemination was performed by the medical staff on the basis of a matching phenotype of the husband. Semen donor was changed after 2 or 3 insemination cycles to a particular woman if pregnancy had not occurred. Donors failing to produce pregnancies were eventually discarded for further use after 25-50 cycles of treatment.

RNA extraction and cDNA synthesis

In order to enrich for fertile spermatozoa and remove somatic contaminants from the expression analysis, the four frozen–thawed semen samples from each donor were individually purified by a centrifugation through discontinuous density gradients (65%–

90%) using a technical procedure similar to that used for IUI (Ruiz-Romero et al., 1995).

Total RNA for each donor was obtained from the pool of the gradient-purified spermatozoa using NucleoSpin® RNA II Kit (Macherey-Nagel, Duren, Germany), according to the instructions provided by the manufacturer with minor modifications. Briefly, lysis buffer was added to the samples at 600 µl/10^7 cells. The lysates were homogenized with a 20-gauge needle and heated for 30 min at 60 ºC. The process then continued with step 4 of the kit, including a DNase digestion step. RNA purity and integrity were assessed by reverse-transcription (RT) of 200 ng of RNA, using a Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and a subsequent PCR using the intron spanning *PRM2* primers as previously described (Ostermeier et al., 2005). As previously shown, the sole existence of the intronless *PRM2* amplicon (148 bp) verified the RNA integrity and showed that the preparations were free of genomic DNA (gDNA) (that would generate a 310 bp intron spanning amplicon). The removal efficiency of somatic and immature germ cell has been confirmed by the absence of *PTPRC* (highly expressed in blood cells), *CDH1* (highly expressed in epithelial cells), and *SYCP3* and *MSH4* (both expressed in meiotic germ cells) expression in our RNA samples (Fig. 2). S*YCP3/MSH4* primer sequences and conditions of amplification were used as published (Terribas et al., 2010). Gene specific primer sets for *PTPRC* (PTPRC-F: aaaagtgcaacgtaatggaagt; PTPRC-R: ccagagtatttccagcttcaac) and for *CDH1* (CDH1-F: ctggttcagatcaaatccaaca; CDH1-R: attggatcctcaactgcattc) were designed by using the Primer3 software (http://frodo.wi.mit.edu/primer3/).

Single-stranded cDNA was obtained by RT of 200 ng of RNA, using the High Capacity cDNA Reverse Transcription Kit (AB, Foster City, California, USA). Two

independent RT reactions were performed from each RNA sample. The resulting cDNA solution was stored at –20ºC until use.

Gene expression quantification

Quantitative real-time PCR assays were performed by means the application of the PCR arrays on micro fluidic cards (MFC), using 384-well TaqMan® Low Density Arrays (TLDAs) on an Applied Biosystems 7900HT Fast Real-Time PCR System (AB, Foster City, California, USA). Half of the RT-reaction was applied on each port, each connecting to 48 reaction wells. A first approach (TLDA1) (Fig. 1) was performed on the 96-gene format MFC (95 experimental assays and 1 TLDA amplification control) allowing simultaneous measurement of 87 target genes that were selected based on human spermatozoa cell location from cDNA microarrays (Ostermeier et al., 2002; Zhao et al., 2006), and 8 ubiquitously expressed genes, commonly used as endogenous control genes to normalize the variability between clinical samples (Vandesompele et al., 2002; De Kok et al., 2005), as potential reference genes for our study. Only samples from donors of the training phase were analysed by this approach. A subsequent second approach (TLDA2) (Fig. 1) was performed on the 24-gene format MFC, which included 21 target genes, 2 reference genes and an amplification control. Both training and validating donor samples were analysed by this approach. For training donors, TLDA2 comprised a different RT reaction of the same donor RNA sample on TLDA1 approach. Genes and the corresponding assays on demand used for the setup of the TLDAs are listed in Supplemental Table I. The design of each assay and its potential gDNA amplification is additionally detailed (Supplemental Table I). Furthemore, in order to check the assay specificity, and thus to verify that the assay can not amplify related processed sequences (i.e. processed retroposed pseudogenes), a Blast analysis on human

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transcript database was performed by using the information of primers and probe sequences of each assay. The primer and probe sequences can be inferred from the assay location (indicates the middle position of the amplicon on the specified RefSeq; this will be the position occupied by the probe), and the amplicon length (5' and 3' sequences of the amplicon will define primer sequences) detailed on the AB assay design (www.appliedbiosystems.com).

Samples from donors with low and high PR were always analysed as paired samples in the same analytical run in order to exclude between-run variations. Real-time PCR data were pre-processed and stored in SDS 2.2 software (AB, Foster City, California, USA).

To confirm reproducibility and precision of real-time PCR experiments, inter-assay variation of samples amplified on both approaches was determined. Variation was measured as the coefficient of variation (CV) of Ct from the Ct mean value of both TLDA approaches. In the above mentioned RT-PCR runs, inter-assay variation ranged from 0.63% to 1.60% with the exception of *PRM1* (2.40%), *PRM2* (2.04%), *ENO1* (4.13%) and *RERE* (3.42%), confirming high reproducibility and precision for most of the 23 genes included in the TLDA1 and TLDA2 approaches.

Data analysis

All statistical analyses were performed using the SPSS version 12 (Lead Technologies, Chicago, USA) software. The nonparametric Kruskal-Wallis test was used to analyze the differences in clinical data, absolute expression levels of reference genes and relative expression of target genes among the study groups 1, 2 and 3 of phase I. Differences in absolute and in relative expression of TLDA1 target genes in patient

group 1 or 2 compared to group 3 were evaluated by the nonparametric Mann-Whitney U test.

Expression stability of the gene/s was calculated with the GeNorm program (Vandesompele et al., 2002), in order to select the most stable reference genes and improve normalization of target genes. GeNorm software calculates the gene expression stability value M of multiple candidate genes as the average pair wise variation of a particular gene compared with all other candidate reference genes. Lower M values indicate genes with less expression variation among samples.

Raw data normalization was performed with the qBase program (Hellemans et al., 2007) by using one reference gene as well as by applying geometric averaging of two reference genes, in parallel. Relative quantification (RQ) values were expressed using the $2^{-\Delta\Delta Ct}$ method as fold changes in the target gene normalized to the reference gene and related to the expression of a control sample. For the training donors–, the mean value of the TLDA1 and TLDA2 normalized $2^{-\Delta\Delta Ct}$ values for each donor were then subjected to evaluation of statistical significance of differential expression among groups (Kruskal-Wallis or Mann-Whitney test as mentioned above).

Pearson product moment correlation coefficients were calculated to correlate the molecular and clinical data of donors. Receiver operating characteristic (ROC) curve analysis of the relative expression values was used for distinguishing those individuals with PR ≤13.6%, which was the cut-off value for 25 percentile. Accuracy was measured as the area under the ROC curve (AUC). The threshold value was determined by Youden's index, calculated as sensitivity plus specificity – 1 (Skendzel and Youden, 1970).

Following the recommendations of the STARD initiative (Bossuyt et al., 2004) we defined 'positives' as donors showing pathological results (i.e. low PR) and thus,

sensitivity corresponded to the proportion of 'infertile donors' successfully detected by a given test (true positive rate), while specificity indicated the 'fertile donors' testing as normal (true negative rate).

Multivariate binary logistic regressions were used for selection of the optimal combination of genes associated with fertilization status of the phase I donors and for validating the combination of genes as a predictive tool in donors of phase II. A backward stepwise (Conditional) method was used to drop insignificant terms. The multivariate regression model included the genes found to significantly distinguish IUI-PR ≤ 13.6%. The binary logistic regression model provides the following estimation of the logit function:

$$Logit(p) = B0 + B1X1 + B2X2 + \ldots.$$

where p=P (adequate fertility potential for insemination), Logit(p)=log(p/(1-p))=log(Odds), B=log OR and Xn= the expression value of the selected genes. Therefore, if we use this estimated model as a prediction model, with the standard classification cutoff of 0.5, we would classify individuals with a positive Logit function estimation as "adequate for insemination" and individuals with negative Logit function estimation as "inadequate for insemination".

Binary logistic regressions of a single genetic variant as well as single/combination of clinical parameters were calculated for comparison of predictive values of the model.

A *p*-value <0.05 was considered significant. False Discovery Rate (FDR) of significant tests was estimated to overcome false positives overestimation in a multiple testing approach (Storey and Tibshirani, 2003).

RESULTS

Reproductive results of semen donors

A total of 545 women underwent 1631 IUI cycles with samples from all 68 donors studied. The PR, live birth rate and miscarriage rate of the donors was 17.21%, 15.2% and 11.8% respectively.

In order to obtain a general overview of the donor gene expression behaviour related to PR outcome that support a further and deeper evaluation of its potential as a diagnostic tool, donors selected for the training phase were first classified into three groups (tertiles) according to the PR obtained after IUI: low –from 0 to 15.70% PR- (group 1), medium –from 15.71 to 23.00% PR- (group 2) and high –from 23.01-45.0% PR- (group 3) (Table I). The average PR of training donors was 18.4%. Although the number of IUI cycles was similar in the three groups, the number of pregnancies, the PR and the live birth rate were significantly different among groups (Table I). Clinical features and baseline semen parameters were similar among tertiles. The average concentration of progressive motile spermatozoa used at the time of inseminations after gradient selection showed a tendency to higher values between groups, with a borderline significance (p=0.058). None of the variables corresponding to the female recipients showed significant differences.

The average PR of the donors included in the validation phase was 15.7%, and live birth rate was 14.4%. These reproductive outcomes, as well as the clinical and analytical variables were comparable to those of the training phase (data not shown).

Selection of genes for the TLDA2 approach

The presence of mRNA for 74 out of the 95 genes of the TLDA1 study (genes in bold, Supplemental table I) was confirmed by RT-PCR in human ejaculated spermatozoa.

The remaining genes (n=21) could not be amplified (Ct value>33) under the conditions of the study suggesting that the transcript levels were beneath the detection threshold of the technique. Of the 74 genes amplified, 35 were excluded for further analysis due to poor amplification efficiency across samples (missing expression values >80%). The mRNA levels of genes amplified in all samples of the study (Supplemental Table I) were further evaluated (n=39) (Fig. 1).

In order to achieve precise and reliable quantitative expression results of the genes under consideration, measurement of gene expression by real-time RT-PCR requires at least one proper internal control reference gene for normalization purposes. None of the eight genes previously ~~described as ubiquitously expressed~~ used as endogenous control genes have quantifiable expression values, so they were excluded as normalizers for our study. Therefore, from the 39 genes we selected those showing stable expression levels in the samples investigated to be subsequently used as normalizers or reference genes. For this purpose, we used the GeNorm program that selected the *RPS17* and *RPL29* as the most stably expressed genes (M= 0.038 for both genes). Additionally, we ascertained that these genes were not differentially expressed among the groups of the study (p>0.05). These two genes were included in the TLDA2 approach as reference genes.

The selection of target genes included in the TLDA2 approach (supplemental table I) was performed taking into account those genes that presented statistical difference in Ct values between group 1 and 3.

Relative gene expression profile of donor sperm

Once the TLDA1 and TLDA2 Ct data from the training donor samples were obtained, the quantification of the 21 target gene mRNA levels was expressed as relative

transcript levels using *RPS17* as a single reference gene as well as the *RPS17* and *RPL29* gene combination reference value for both TLDA experiments.

When training donors were classified into tertiles according to the IUI PR, we found eight differentially expressed genes among the three groups: *RPL23A, RPS27A, RPS8* ($p \leq 0.01$)*, RBM9, RPS27, RPS3, TOMM7* and *RPS18* ($p \leq 0.05$), when normalized with both single and combination of reference genes (Fig. 3). The FDR value of significant tests was quite small (1.7%). All of them presented small intra-group standard deviation values (0.08-0.30).

RPL23A, RPS27A, RPS3, RPS8, TOMM7 genes showed a significant fold-change decrease in group 1 of 1.22 (p=0.001), 1.39 (p=0.0002), 1.22 (p=0.004), 1.13 (p=0.005), and 1.26 (p=0.026) respectively when compared to group 3 (Fig. 3). The FDR value was 0.082 implying that 8.2% of significant tests will result in false positives. Interestingly, *RPL23A*, *RPS27A, RPS3*, *RPS8* and *TOMM7* showed a linear tendency among the three groups of the study.

Since, data normalization using both single and combination of reference genes resulted in the same statistical data; gene expression data normalized with *RPS17* were subsequently used to simplify the model.

Correlation study between gene expression profiles and semen parameters or PR

No significant correlation was found between the sperm baseline concentration or motility semen parameters and the relative mRNA expression levels of any of the 21 genes analysed. However, morphology of spermatozoa was found to be positively correlated with *FOXG1* (r:0.341; p=0.025) and *RPS8* (r:0.371; p=0.014) transcript levels. When assessing the sperm parameters post-thaw, significant correlations were found between the percentage of post-thaw motile sperm and *EIF5A* (r:0.355; p=0.019),

382 *RPL13* (*r*:0.397; *p*=0.008), *RPL23A* (*r*:0.346; *p*=0.023), *RPL7* (*r*:-0.353; *p*=0.020),
383 *RPS18* (*r*:-0.390; *p*=0.010) and *RPS6* (*r*:-0.337; *p*=0.027) as well as between the post-
384 thaw motile sperm count and *RPS18* (*r*:-0.326; *p*=0.033) and *RPS27* (*r*:-0.345; *p*=0.024)

385 In order to investigate a possible association between gene expression and the
386 PR and to confirm whether the results could be of physiological and/or clinical
387 relevance, we performed a correlation study between the normalized gene expression
388 ratios and the PR mean value of the insemination cycles in which the donor sample was
389 used. Significant positive correlation coefficients were found between PR and the
390 transcription levels of six genes: *RPL23A*, *RPL4*, *RPS27A*, *RPS3*, *RPS8* and *TOMM7*
391 ($p<0.05$) (Table II). We performed the same type of analysis for other clinical
392 reproductive parameters such as the birth rate and the miscarriage rate. Three additional
393 genes: *RPL10A*, *RPS6* and *RBM9* expression values were found to significantly
394 correlate with miscarriage rate ($p\leq0.05$) (Table II). Similar correlation studies were
395 additionally performed using semen parameters for comparison (Table II).

396 We hypothesized that there might be a threshold level of transcripts with the
397 potential for discriminating donors with lower PR. We then selected as the state variable
398 the 25th percentile of PR produced by the donors of the training phase, which was ≤13.6
399 %. The ROC curve analysis of gene expression levels resulted in good predictive
400 accuracy (AUC>0.750) of the expression values of seven genes: *EIF5A*, *RPL13*,
401 *RPL23A*, *RPS27A*, *RPS3*, *RPS8* and *TOMM7* ($p<0.01$). Therefore, they were selected as
402 potential genetic biomarkers of sperm function (Table III). None of the classical semen
403 parameters reached AUC significantly higher than 0.5. The mean of progressively
404 motile sperm inseminated values were excluded from the analysis because this
405 information was obtained at the time of IUI treatment, and thus could not be used as a
406 tool for screening the future fertility of the donors.

Searching for a multiplex model: multivariate logistic regression analysis

To determine if a multiplex model could improve performance over single biomarkers for discriminating donors with ≤13.6% PR, the previously selected genes were analyzed in a multivariate regression analysis.

This analysis resulted in a model that included *EIF5A*, *RPL13*, *RPL23A* and *RPS27A* genes (Table IV). The sensitivity and the specificity for predicting donors with low IUI PR were 90% and 97%, respectively. The accuracy of the test was corroborated as the calculated AUC was 0.955 (p=0.000) and the p-value of Hosmer and Lemeshow test was 0.554.

As comparison, a multivariate regression analysis of all the semen parameters was performed. The resulting model, including the combination of the baseline percentage of progressive motility and post-thaw progressive motility count variables, resulted in a sensitivity of 30%, specificity of 94% and AUC of 0.773 (p=0.010) (Table IV).

The classifier based on gene expression values of phase I donors was validated in samples of donors of phase II, resulting in a sensitivity of 71.5%, a specificity of 78% and accuracy of 76% (Table IV). Identification of positive individuals (infertile donors) was more~~less~~ efficient when applying the classifier than~~that~~ resulted when using the semen values. The model with semen parameters in phase II donors showed a sensitivity of 14.3% and a specificity of 100% (Table IV).

When the results of all donors were considered together, the multivariate logistic analysis using the genetic markers showed a true positive rate or sensitivity of~~was~~ 82.3% (14/17), compared with 23.5% (4/17) obtained when using the semen variables. As a consequence, 10 additional positive donors (58.8%) could be detected by using the

gene signature. From another point of view, the ability to identify false negatives (subfertile donors testing as normal) was better with the genetic approach: the probability of normality [p(D-)] after a negative result increased to 93.9% from a pre-test probability of 75% (incremental gain of 18.9%), whereas p(D-) gain was only 4% with semen parameters (Fig. 4).

DISCUSSION

Despite having normal sperm parameters, differences in the fecundity of donor sperm are observed after insemination therapies (Marshburn et al., 1992; Navarrete et al., 2000; Thyer et al., 1999). Classical sperm variables at the time of baseline evaluation of donors have limited value for predicting their reproductive fitness for insemination therapies (Freour et al., 2009; Sidhu et al., 1997). Thus, close supervision of the clinical results obtained by each donor is the only pragmatic way to discard those who show poor PR after a reasonable number of insemination cycles (Johnston et al., 1994). Some studies suggest that characteristics of the thawed samples used in the insemination (number of motile sperm, kinetic or morphometric parameters measured with CASA systems) are able to improve the accuracy of pregnancy outcome (Freour et al., 2009; Macleod and Irvine, 1995; Marshburn et al., 1992) in comparison with the baseline assessments. However, these measurements made on individual samples are of limited utility for the purpose of deciding if a candidate man can be accepted as a valid semen donor. Other approaches to improve the assessment of donor fecundity are based on multiparametric analysis of semen characteristics (Allamaneni et al., 2004) or a number of sperm function tests (Richardson and Aitken, 1993). However, the diagnostic performance of these methods is modest due to the low sensitivity of the results obtained.

In this report, we have developed a molecular means to classify the fertility status of semen donors for IUI based on gene expression profiles of sperm. Our experimental approach was the selection of the gene set expression panel in a training series of semen donors with a detailed record of IUI reproductive outcome in different female recipients. The potential of the gene panel as a predictive classifier was validated in an independent series of donors. The sensitivity value of the genetic model (*EIF5A*,

RPL13, *RPL23A* and *RPS27A* genes) as a predictive tool for discriminating donors with low IUI PR was far better than that obtained from the combination of traditional sperm parameters. As a result, 58.8% of positive (subfertile) donors effectively detected by the gene signature approach were wrongly classified as negative (fertile) with the semen variables. It is generally accepted that a diagnostic test for male infertility in couples attempting pregnancy should have high specificity, reducing false positives (fertile men testing as abnormal) in order to avoid the over-treatment of couples with aggressive and costly techniques such as IVF and ICSI. However, in the case of deciding if a donor with normal semen parameters is likely to produce pregnancy the situation changes. In this case all parties involved in the process of artificial insemination with donor sperm are interested in achieving the highest possible pregnancy rates. Therefore it is preferable to have a test with high sensitivity, identifying those donors that produce low PR to prevent the subsequent use of their semen samples, even at the price of discarding few fertile donors if specificity becomes suboptimal. A similar scenario is found in couples with unexplained infertility, although in this case the expected probability of male subfertility is likely to be higher. There is insufficient evidence to know the relative effectiveness of intrauterine insemination and IVF/ICSI in couples with unexplained fertility problems (NICE, 2004). Although husband IUI has been widely used as an empirical treatment for unexplained infertility (Verhulst et al, 2006) some couples with hidden defects in sperm function will not become pregnant, and will have to undergo IVF/ICSI. Conversely, it is possible that low-tech treatment would be enough to produce pregnancy to some of those patients who go directly to IVF/ICSI (Collins, 2003). Hence, a prognostic marker to estimate the chances for fertility treatment could help physicians in counseling about the best treatment for patients. In this context, the test described here would be able to select more men who show

reduced fertility potential, and will benefit from early referral to IVF/ICSI, avoiding unsuccessful alternatives such as expectant management or husband IUI.

When the IUI technique is coupled with ovarian stimulation, the sperm cells are placed directly into the uterus at the time of ovulation, bypassing ovulatory alterations and cervical hostility in the recipient women as confounding factors. The effect of additional female factors has been well recognized, and can heavily influence the reproductive outcome of donor insemination (Botchan et al., 2001; De Brucker et al., 2009). In our study design, the use of multiple female recipients, with no significant differences in age and gynaecological conditions between the groups with diverse PR, reduced the impact of female factors as confounding elements on the donor reproductive outcome. Therefore, we believe that the reproductive differences observed are truly representative of intrinsic properties of the sperm used, which tend to be constant for each donor over the time (Thyer et al., 1999).

The quality of spermatozoal RNA used in this study, a big concern to studies like this one, had been further examined. Firstly, the absence of genomic fragments in the RT-PCR result for *PRM2* confirmed that DNA contamination (visible in some samples when studying *PTPRC*) is very low, and therefore, unlikely to affect the quantification of strongly transcribed genes such as those studied in this work. Furthermore, the removal efficiency of somatic cells was also taken into account. The lack of amplification of ~~the ubiquitously expressed~~ genes such as *HPRT*, *HMBS*, *PGM1*, *GUSB*, *PGK1* and *TBP,* included in the TLDA1 approach, suggest that these genes, widely used as endogenous genes in somatic tissues (Vandesompele et al., 2002; De Kok et al., 2005), are poorly expressed in spermatozoa. As far as we know, there is no previous data that suggest expression of these genes in sperm. Altogether, it is an

additional indication that the somatic cell contamination is not elevated enough to affect spermatozoal transcript quantification.

One of the major drawbacks for the clinical use of sperm gene expression data is the low level of transcripts contained in spermatozoa. PCR amplification on TLDAs was considered an appropriate method for our study and for future potential diagnostic purposes, because it allows the simultaneous quantitative amplification of multiple reactions with minimal cDNA material and a reduced variability due to pipetting. Furthermore, in order to improve experimental accuracy, data were normalized to suitable reference genes, which showed constitutive and stable expression levels in the samples investigated.

Expression differences were previously found in sperm related to spermatozoa motility and capacitation (Lambard et al., 2004) and in sperm transcriptomes from fertile vs. idiopathic infertile men (Avendano et al., 2009; Garrido et al., 2009). To the best of our knowledge, our study is the first attempt to systematically explore the diagnostic possibilities of sperm mRNA expression patterns. We observed significant differences in expression of some individual genes *RPL23A, RPS27A, RPS3, RPS8, TOMM7* between sperm samples from donors with the worst and the best PR after IUI assisted reproduction. Interestingly, one of these genes, *RPS3,* was found differentially expressed between men of proven fertility and men with idiopathic infertility in a previous study (Garrido et al., 2009). Hence, description of the normozoospermic sperm transcriptome could also be helpful to identify genes or gene pathways responsible for idiopathic infertility.

In conclusion, our findings contribute to the task of selecting the best genetic markers for use for the prediction of the fecundity ability of spermatozoa. This will lead to an improvement in the pregnancy outcome of the assisted reproduction. We have

shown an expression fingerprint related to the fertilizing ability of sperm when used in therapeutic IUI that could complement semen analysis as a fertility test with several therapeutic uses. These include selection of those samples from donor semen banks appropriate for use for IUI assisted reproduction as well as provision of realistic information about the chances of success of conjugal IUI for couples with unexplained infertility.

AUTHOR'S ROLES

S. B. was involved in molecular genetics work and data analysis, A. M. was responsible for sample selection and clinical data, M.D. R participated in the performance of the molecular genetics experiments, Ll. B. participated in the design of the study, sample selection and helped to draft the manuscript, and S. L. designed the study, coordinated its development, supervised the molecular genetics work and drafted the manuscript

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562 **REFERENCES**

563

564 Allamaneni, S. S., Bandaranayake, I. and Agarwal, A. Use of semen quality scores to
565 predict pregnancy rates in couples undergoing intrauterine insemination with donor
566 sperm. *Fertil Steril* 2004; **82**: 606-611.
567 Avendano, C., Franchi, A., Jones, E. and Oehninger, S. Pregnancy-specific {beta}-1-
568 glycoprotein 1 and human leukocyte antigen-E mRNA in human sperm: differential
569 expression in fertile and infertile men and evidence of a possible functional role during
570 early development. *Hum Reprod* 2009; **24**: 270-277.
571 Bartoov, B., Eltes, F., Pansky, M., Lederman, H., Caspi, E. and Soffer, Y. Estimating
572 fertility potential via semen analysis data. *Hum Reprod* 1993; **8**: 65-70.
573 Boerke, A., Dieleman, S. J. and Gadella, B. M. A possible role for sperm RNA in early
574 embryo development. *Theriogenology* 2007; **68 Suppl 1**: S147-155.
575 Bossuyt, P. M., Reitsma, J. B., Bruns, D. E., Gatsonis, C. A., Glasziou, P. P., Irwig, L.
576 M., Lijmer, J. G., Moher, D., Rennie, D. and de Vet, H. C. Towards complete and
577 accurate reporting of studies of diagnostic accuracy: the STARD initiative. *Fam Pract*
578 2004; **21**: 4-10.
579 Botchan, A., Hauser, R., Gamzu, R., Yogev, L., Paz, G. and Yavetz, H. Results of 6139
580 artificial insemination cycles with donor spermatozoa. *Hum Reprod* 2001; **16**: 2298-
581 2304.
582 Braude, P., Bolton, V. and Moore, S. Human gene expression first occurs between the
583 four- and eight-cell stages of preimplantation development. *Nature* 1988; **332**: 459-
584 461.
585 Carreau, S., Lambard, S., Said, L., Saad, A. and Galeraud-Denis, I. RNA dynamics of
586 fertile and infertile spermatozoa. *Biochem Soc Trans* 2007; **35**: 634-636.

587 Collins J. Stimulated intra-uterine insemination is not a natural choice for the treatment
588 of unexplained subfertility. Current best evidence for the advanced treatment of
589 unexplained subfertility. *Hum Reprod* 2003; **18**:907-912
590 Cooper, T. G., Noonan, E., von Eckardstein, S., Auger, J., Baker, H. W., Behre, H. M.,
591 Haugen, T. B., Kruger, T., Wang, C., Mbizvo, M. T. et al. World Health Organization
592 reference values for human semen characteristics. *Hum Reprod Update* 2010; **16**: 231-
593 245.
594 De Brucker, M., Haentjens, P., Evenepoel, J., Devroey, P., Collins, J. and Tournaye, H.
595 Cumulative delivery rates in different age groups after artificial insemination with
596 donor sperm. *Hum Reprod* 2009; **24**: 1891-1899.
597 De Kok, J. B., Roelofs, R. W., Giesendorf, B. A., Pennings, J. L., Waas, E. T., Feuth,
598 T., Swinkels, D. W. and Span P. N. Normalization of gene expression measurements
599 in tumor tissues: comparison of 13 endogenous control genes. *Lab Invest* 2005; **85**:
600 154-159.
601 Freour, T., Jean, M., Mirallie, S., Langlois, M. L., Dubourdieu, S. and Barriere, P.
602 Predictive value of CASA parameters in IUI with frozen donor sperm. *Int J Androl*
603 2009; **32**: 498-504.
604 Garrido, N., Martinez-Conejero, J. A., Jauregui, J., Horcajadas, J. A., Simon, C.,
605 Remohi, J. and Meseguer, M. Microarray analysis in sperm from fertile and infertile
606 men without basic sperm analysis abnormalities reveals a significantly different
607 transcriptome. *Fertil Steril* 2009; **91**: 1307-1310.
608 Guo, X., Gui, Y. T., Tang, A. F., Lu, L. H., Gao, X. and Cai, Z. M. Differential
609 expression of VASA gene in ejaculated spermatozoa from normozoospermic men and
610 patients with oligozoospermia. *Asian J Androl* 2007; **9**: 339-344.

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611 Gur, Y. and Breitbart, H. Mammalian sperm translate nuclear-encoded proteins by
612 mitochondrial-type ribosomes. *Genes Dev* 2006; **20**: 411-416.
613 Guzick, D. S., Overstreet, J. W., Factor-Litvak, P., Brazil, C. K., Nakajima, S. T.,
614 Coutifaris, C., Carson, S. A., Cisneros, P., Steinkampf, M. P., Hill, J. A. et al. Sperm
615 morphology, motility, and concentration in fertile and infertile men. *N Engl J Med*
616 2001; **345**: 1388-1393.
617 Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. and Vandesompele, J. qBase
618 relative quantification framework and software for management and automated
619 analysis of real-time quantitative PCR data. *Genome Biol* 2007; **8**: R19.
620 Johnston, R. C., Kovacs, G. T., Lording, D. H. and Baker, H. W. Correlation of semen
621 variables and pregnancy rates for donor insemination: a 15-year retrospective. *Fertil*
622 *Steril* 1994; **61**: 355-359.
623 Krawetz, S. A. Paternal contribution: new insights and future challenges. *Nat Rev Genet*
624 2005; **6**: 633-642.
625 Lambard, S., Galeraud-Denis, I., Martin, G., Levy, R., Chocat, A. and Carreau, S.
626 Analysis and significance of mRNA in human ejaculated sperm from
627 normozoospermic donors: relationship to sperm motility and capacitation. *Mol Hum*
628 *Reprod* 2004; **10**: 535-541.
629 Lewis, S. E. Is sperm evaluation useful in predicting human fertility? *Reproduction*
630 2007; **134**: 31-40.
631 Macleod, I. C. and Irvine, D. S. The predictive value of computer-assisted semen
632 analysis in the context of a donor insemination programme. *Hum Reprod* 1995; **10**:
633 580-586.

634 Marshburn, P. B., McIntire, D., Carr, B. R. and Byrd, W. Spermatozoal characteristics
635 from fresh and frozen donor semen and their correlation with fertility outcome after
636 intrauterine insemination. *Fertil Steril* 1992; **58**: 179-186.
637 Miller, D. and Ostermeier, G. C. Towards a better understanding of RNA carriage by
638 ejaculate spermatozoa. *Hum Reprod Update* 2006; **12**: 757-767.
639 Miteva, K., Valkov, N., Goncharova-Peinoval, J., Kovachev, K., Zlatarev, S.,
640 Pironcheva, G. and Russev, G. Electron microscopic data for the presence of post-
641 meiotic gene expression in isolated ram sperm chromatin. *Cytobios* 1995; **83**: 85-
642 90.Navarrete, T., Johnson, A., Mixon, B. and Wolf, D. The relationship between
643 fertility potential measurements on cryobanked semen and fecundity of sperm donors.
644 *Hum Reprod* 2000; **15**: 344-350.
645 Naz, R. K. Effect of actinomycin D and cycloheximide on human sperm function. *Arch*
646 *Androl* 1998; **41**: 135-142.
647 NICE, National Collaborating Centre for Women's and Children's Health
648 Commissioned by the National Institute for Clinical Excellence. *Fertility: assessment*
649 *and treatment for people with fertility problems. Clinical guideline*. RCOG Press,
650 London, 2004
651 Ostermeier, G. C., Dix, D. J., Miller, D., Khatri, P. and Krawetz, S. A. Spermatozoal
652 RNA profiles of normal fertile men. *Lancet* 2002; **360**: 772-777.
653 Ostermeier, G. C., Miller, D., Huntriss, J. D., Diamond, M. P. and Krawetz, S. A.
654 Reproductive biology: delivering spermatozoan RNA to the oocyte. *Nature* 2004; **429**:
655 154.
656 Ostermeier, G. C., Goodrich, R. J., Diamond, M. P., Dix, D. J. and Krawetz, S. A.
657 Toward using stable spermatozoal RNAs for prognostic assessment of male factor
658 fertility. *Fertil Steril* 2005; **83**: 1687-1694.

659 Pedigo, N.G., Vernon, M.W. and Curry, T.E. Jr. Characterization of a computerized
660 semen analysis system. *Fertil Steril* 1989; **52**: 659-666.
661 Platts, A. E., Dix, D. J., Chemes, H. E., Thompson, K. E., Goodrich, R., Rockett, J. C.,
662 Rawe, V. Y., Quintana, S., Diamond, M. P., Strader, L. F. et al. Success and failure in
663 human spermatogenesis as revealed by teratozoospermic RNAs. *Hum Mol Genet*
664 2007; **16**: 763-773.
665 Richardson, D. W. and Aitken, R. J. *Techniques for examining the fertilizing capacity*
666 *of semen.* 1993. Cambridge University Press.
667 Ruiz-Romero, J., Antich, M. and Bassas, L. Choosing among different technical
668 variations of Percoll centrifugation for sperm selection. *Andrologia* 1995; **27**: 149-153.
669 Samplaski, M. K., Agarwal, A., Sharma, R. and Sabanegh, E. New generation of
670 diagnostic tests for infertility: review of specialized semen tests. *Int J Urol* 2010; **17**:
671 839-847.
672 Sidhu, R. S., Sharma, R. K. and Agarwal, A. Effects of cryopreserved semen quality
673 and timed intrauterine insemination on pregnancy rate and gender of offspring in a
674 donor insemination program. *J Assist Reprod Genet* 1997; **14**: 531-537.
675 Siffroi, J. P. and Dadoune, J. P. Accumulation of transcripts in the mature human sperm
676 nucleus: implication of the haploid genome in a functional role. *Ital J Anat Embryol*
677 2001; **106**: 189-197.
678 Skendzel, L. P. and Youden, W. J. Systematic versus random error in laboratory
679 surveys. *Am J Clin Pathol* 1970; **54**: 448-450.
680 Steger, K., Wilhelm, J., Konrad, L., Stalf, T., Greb, R., Diemer, T., Kliesch, S.,
681 Bergmann, M. and Weidner, W. Both protamine-1 to protamine-2 mRNA ratio and
682 Bcl2 mRNA content in testicular spermatids and ejaculated spermatozoa discriminate
683 between fertile and infertile men. *Hum Reprod* 2008; **23**: 11-16.

684 Storey, J.D. and Tibshirani, R. Statistical significance for genomewide studies. *Proc*
685 *Natl Acad Sci U S A*. 2003; **100**: 9440-9445.
686 Terribas, E., Bonache, S., Garcia-Arévalo, M., Sanchez, J., Franco, E., Bassas, L. and
687 Larriba, S. Changes in the expression profile of the meiosis-involved mismatch repair
688 genes in impaired human spermatogenesis. *J Androl* 2010; **31**: 346-357
689 Thyer, A. C., Patton, P. E., Burry, K. A., Mixon, B. A. and Wolf, D. P. Fecundability
690 trends among sperm donors as a measure of donor performance. *Fertil Steril* 1999; **71**:
691 891-895.
692 Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and
693 Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by
694 geometric averaging of multiple internal control genes. *Genome Biol* 2002; **3**:
695 RESEARCH0034.
696 Verhulst, S.M., Cohlen, B.J., Hughes, E., Te Velde, E., Heineman and M.J. Intra-uterine
697 insemination for unexplained subfertility. Cochrane Database *Syst Rev* 2006; Oct
698 18;(4):CD001838.
699 WHO, World Health Organization *Laboratory Manual for the Examination of Human*
700 *Semen and Sperm-Cervical Mucus Interaction*. 4th edn. edn1999. Cambridge
701 University Press, New York.
702 Zhao, Y., Li, Q., Yao, C., Wang, Z., Zhou, Y., Wang, Y., Liu, L., Wang, Y., Wang, L.
703 and Qiao, Z. Characterization and quantification of mRNA transcripts in ejaculated
704 spermatozoa of fertile men by serial analysis of gene expression. *Hum Reprod* 2006;
705 **21**: 1583-1590.
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Table I Clinical characteristics of semen donors and female recipients (training phase) classified by tertiles according to pregnancy rates (PR) after intrauterine inseminations (mean ± SD)

	Group 1 n=14	Group 2 n=14	Group 3 n=15	*P*-value
Semen donors				
Insemination cycles per donor (n)	23.71±16.9	20.57±9.3	19.53±8.8	0.858[§]
Pregnancies per donor (n)	2.21±1.9	3.64±1.7	5.73±2.7	**0.0001**[§]
Pregnancy rate (%)[α]	9.30 (6.4-12.9)	17.70 (13.5-22.6)	29.30 (24.2-34.9)	**<0.0001**[#]
Live birth rate (%)[α]	9.03 (6.2-12.6)	15.27 (11.3-19.9)	23.89 (19.1-29.2)	**<0.0001**[#]
Age (years)	24.57±4.8	25.71±5.0	25.53±5.2	0.813[§]
BMI	23.03±2.4	22.93±2.4	23.23±2.5	0.982[§]
Semen volume (mL)	3.49±0.9	3.82±0.8	3.71±1.5	0.726[§]
Sperm count (x10^6/mL)	79.21±36.8	101.75±39.9	87.23±34.8	0.166[§]
Progressive motility (%)	59.18±11.4	58.36±10.3	57.07±10.5	0.814[§]
VAP (µm/sec)	30.27±6.7*	31.68±5.7**	30.00±4.8***	0.829[§]
VSL (µm/sec)	23.32±5.7*	24.77±4.1**	23.61±4.0***	0.907[§]
STR (%)	75.00±4.2*	75.00±3.9**	73.14±8.8***	0.956[§]
ALH (µm)	4.04±1.3*	4.10±1.0**	4.18±1.0***	0.901[§]
Normal morphology (%)	23.53±10.5	26.31±11.5	24.60±8.8	0.936[§]
Post thaw progressive motility (%)	33.71±10.5	38.33±10.4	39.95±9.1	0.245[§]
Post thaw progressive motile sperm count (x10^6/mL)	13.63±7.9	20.73±12.2	17.48±7.6	0.159[§]
Progressive motile sperm inseminated (x10^6/mL)	12.43±7.3	15.98±6.5	17.99±6.2	0.058[§]
Female recipients				
Average number per donor (n)	14.50±8.3	14.29±6.5	12.94±5.3	0.872[§]
Age (years)	34.69±1.7	35.40±1.4	34.78±1.0	0.461[§]
BMI	25.13±2.0	24.68±1.3	25.39±1.6	0.339[§]
Normal gynecological assessment (%)[α]	72.9 (66.2-78.8)	75.5 (68.9-81.3)	74.2 (67.5-80.2)	0.360[#]
Miscarriage rate per donor (%)	12.50±27.1	11.6±13.3	15.60±11.2	0.183[§]

BMI: body mass index; *n*: number of each trait; *VAP*: average-path velocity; *VSL*: straight-line velocity; *STR*: straightness index; *ALH:* amplitude of lateral head displacement

[α] proportion within group (95% confidence interval)

*n=11, **n=12, ***n=14

[§]Kruskal-Wallis test; [#]Contingency three-by-two tables with significance calculation using Yates correction of chi-square test and the Fisher test to the 5% limit. Significant differences ($p<0.05$) are indicated in bold.

720 **Table II** Pearson correlation coefficients and adjusted *p*-values (r; *p*) between the expression
721 ratios of the target genes and the pregnancy rates after inseminations for phase I donors.
722 Correlation between sperm clinical parameters and pregnancy rates are also shown for
723 comparison. Significant differences ($p \leq 0.05$) are indicated in bold. PR: pregnancy rate, IUI:
724 intrauterine insemination; PM: progressive motility.

Gene expression values			Sperm clinical parameters		
	IUI PR	Miscarriage rate		IUI PR	Miscarriage rate
RPL23A	0.467; *p*=**0.002**	0.110; *p*=0.483	Sperm count (x10^6/mL)	0.099; *p*=0.527	-0.043; *p*=0.783
RPL4	0.419; *p*=**0.005**	0.055; *p*=0.726	Progressive motility (%)	- 0.073; *p*=0.641	0.050; *p*=0.751
RPS27A	0.530; *p*=**0.000**	0.206; *p*=0.185	Normal sperm morphology (%)	0.153; *p*=0.329	-0.035; *p*=0.826
RPS3	0.463; *p*=**0.002**	0.103; *p*=0.509	Post-thaw PM (%)	0.184 *p*=0.237	-0.096 *p*=0.542
RPS8	0.450; *p*=**0.002**	-0.014; *p*=0.930	Post-thaw PM count (x10^6/mL)	0.152 *p*=0.330	-0.070 *p*=0.656
TOMM7	0.308; *p*=**0.044**	0.189; *p*=0.225			
RPL10A	0.283; *p*=0.066	0.403; *p*=**0.007**			
RPS6	0.086; *p*=0.584	0.326; *p*=**0.033**			
RBM9	0.145; *p*=0.352	-0.301; *p*=**0.050**			

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Table III ROC analysis showing the predictive efficiency of seminal and genetic variables for discriminating donors with low IUI pregnancy rates (<13.6%)

Variables	AUC	95% CI	*p*-value
Semen concentration	0.588	0.363-0.813	0.404
Progressive motility (%)	0.348	0.141-0.556	0.151
Normal morphology (%)	0.615	0.399-0.831	0.275
Post-thaw PM (%)	0.598	0.416-0.781	0.350
Post thaw PM count	0.594	0.390-0.798	0.373
VAP	0.448	0.181-0.715	0.658
VSL	0.433	0.174-0.693	0.567
STR	0.373	0.167-0.579	0.276
ALH	0.595	0.360-0.830	0.417
EIF5A	0.827	0.691-0.964	**0.002**
ENO1	0.470	0.253-0.686	0.774
FOXG1	0.632	0.428-0.835	0.211
PRM1	0.500	0.280-0.720	1.000
PRM2	0.512	0.298-0.726	0.908
RBM9	0.415	0.213-0.618	0.421
RERE	0.598	0.382-0.815	0.350
RPL10A	0.662	0.464-0.860	0.124
RPL13	0.859	0.724-0.994	**0.001**
RPL23A	0.882	0.752-1.000	**0.000**
RPL4	0.683	0.498-0.868	0.082
RPL7	0.392	0.223-0.562	0.307
RPS13	0.432	0.234-0.630	0.518
RPS18	0.321	0.150-0.492	0.090
RPS27	0.380	0.211-0.550	0.256
RPS27A	0.894	0.798-0.990	**0.000**
RPS29	0.436	0.244-0.629	0.546
RPS3	0.788	0.642-0.934	**0.006**
RPS6	0.576	0.351-0.800	0.472
RPS8	0.770	0.586-0.954	**0.011**
TOMM7	0.870	0.748-0.992	**0.000**

AUC: area under the curve; CI: confidence interval; PM: progressive motility; VAP: average-path velocity; VSL: straight-line velocity; STR: straightness; ALH: amplitude of lateral head displacement

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Table IV Diagnostic efficiency of multivariate logistic analysis including selected semen and genetic variables in the different phases of the study.

	Phase I	Phase II	Phase I+II
Semen variables			
(PM % + CPM-PT)			
Sensitivity	30	14.3	23.5
Specificity	94	100	96.1
Positive predictive value	60	100	66.6
Negative predictive value	81	75	79
Incremental gain of p(D+)	35	75	41.6
Incremental gain of p(D-)	6	0	4
Gene expression variables			
(*EIF5A* + *RPL13* + *RPL23A* + *RPS27A)*			
Sensitivity	90	71.4	82.3
Specificity	97	77.8	90.2
Positive predictive value	90	55.5	73.7
Negative predictive value	97	87.5	93.9
Incremental gain of p(D+)*	65	30.5	48.7
Incremental gain of p(D-)**	22	12.5	18.9

PM %: percentage of baseline progressive motility
CPM-PT: concentration of post-thaw progressive motile sperm
p(D+): post-test probability with positive result
p(D-): post-test probability with negative result
* Considering pre-test probability of disease (low pregnancy)=0.25
** Considering pre-test probability of normallity (good pregnancy)=0.75

731

732

Figure legends

Figure 1 Flow chart representing the number of individuals and candidate genes through the different work-procedure stages.

Figure 2 . RT-PCR analysis of control transcripts for spermatozoal RNA purity and integrity assessment. **A.B.C.E.** 5ul of amplified product on a 1% agarose gel and **D.** 2.5% agarose gel (nusieve/agarose 3:1). *M*: molecular weight marker, *lanes 1-4*: cDNA from spermatozoa, *lane 5a*: control testicular cDNA, *lane 5b:* cDNA control from lymphocytes, *lane 5c:* cDNA control from colon, *lane 6*: control DNA from lymphocytes and *lane 0*: water (negative control). *PRM2* and *PTPRC* primers allow the amplification of both cDNA and gDNA .

Figure 3

Expression ratios of target genes, using *RPS17* as normalizer, in training donors classified into tertiles according to the IUI PR. Genes with significant differences between groups ($p<0.05$, Kruskal-Wallis test) are displayed in panel (A), and those with non significant changes are shown in panel (B).

Group 1, black bars; group 2, grey bars and group 3, white bars. The gene expression mean value (bars) and 95% CI (error bars Y) are shown.

Figure 4 ROC curves for predictive classification of all the donors studied (phase I + II) using the models developed by multivariate logistic analysis. The area under the curve (AUC) for the model based on semen variables (dashed line) was 0.729 (0.576-0.882), and for the gene expression model (solid line) was 0.910 (0.836-0.984)

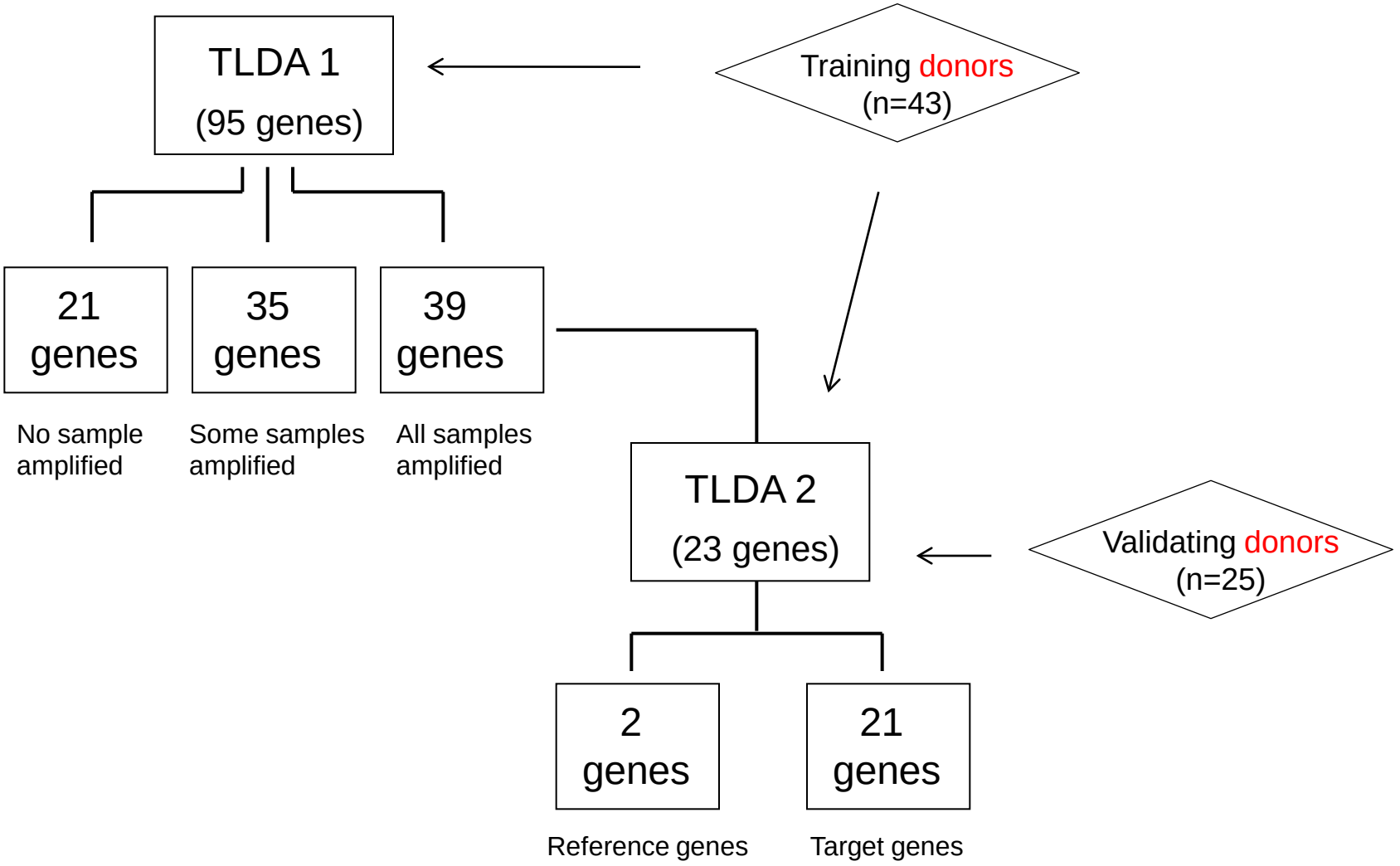
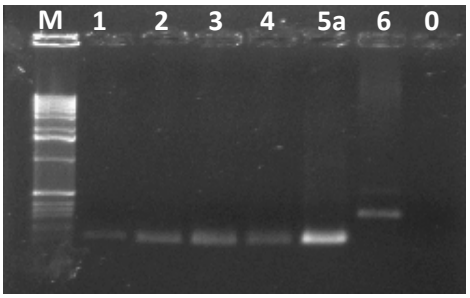
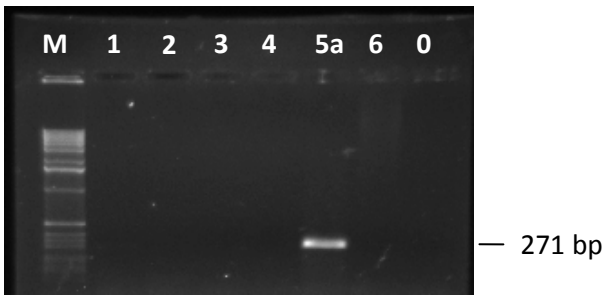

TLDA 1
(95 genes)
Training donors
(n=43)
21 genes
35 genes
39 genes
No sample amplified
Some samples amplified
All samples amplified
TLDA 2
(23 genes)
Validating donors
(n=25)
2 genes
21 genes
Reference genes
Target genes

Figure 2

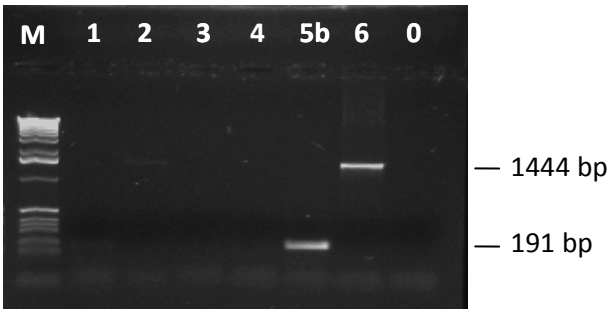
A. PRM2 (Exon 1 ->2)



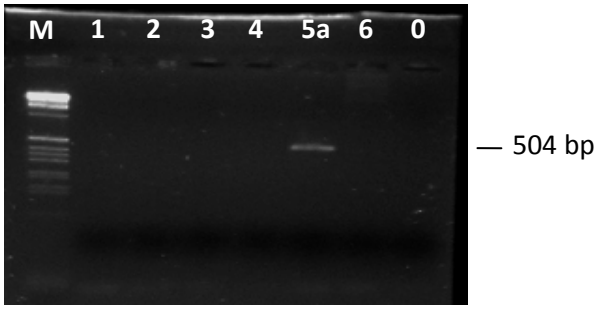
C. SYCP3 (Exon 2/3 ->6)



B. PTPRC (Exon 11 -> 12)



D. MSH4 (Exon 2/3 -> 6)



E. CDH1 (Exon 4/5 -> 6)

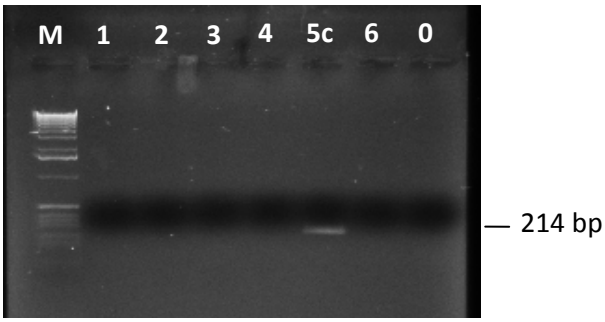
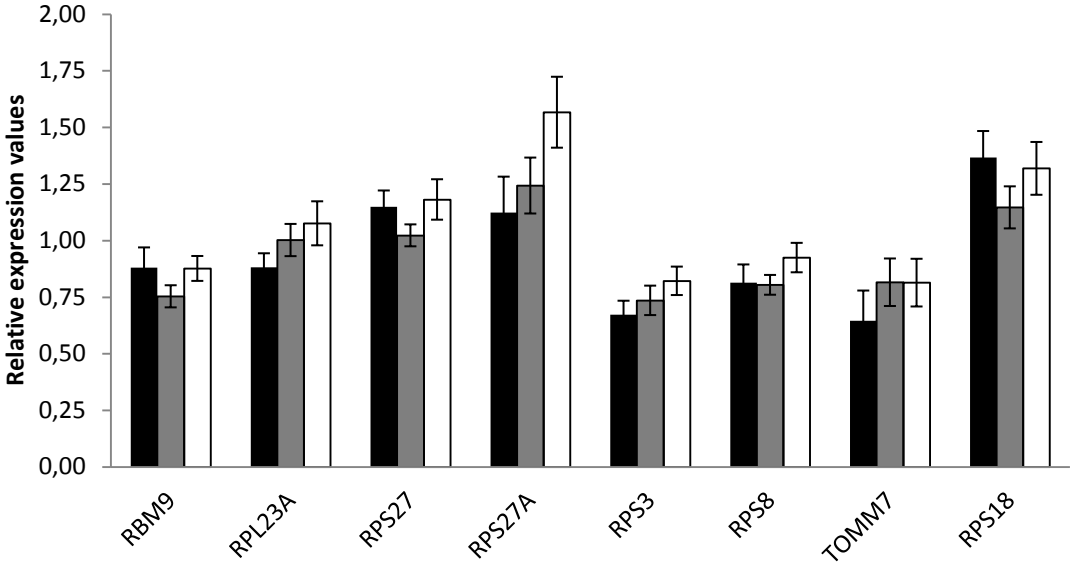
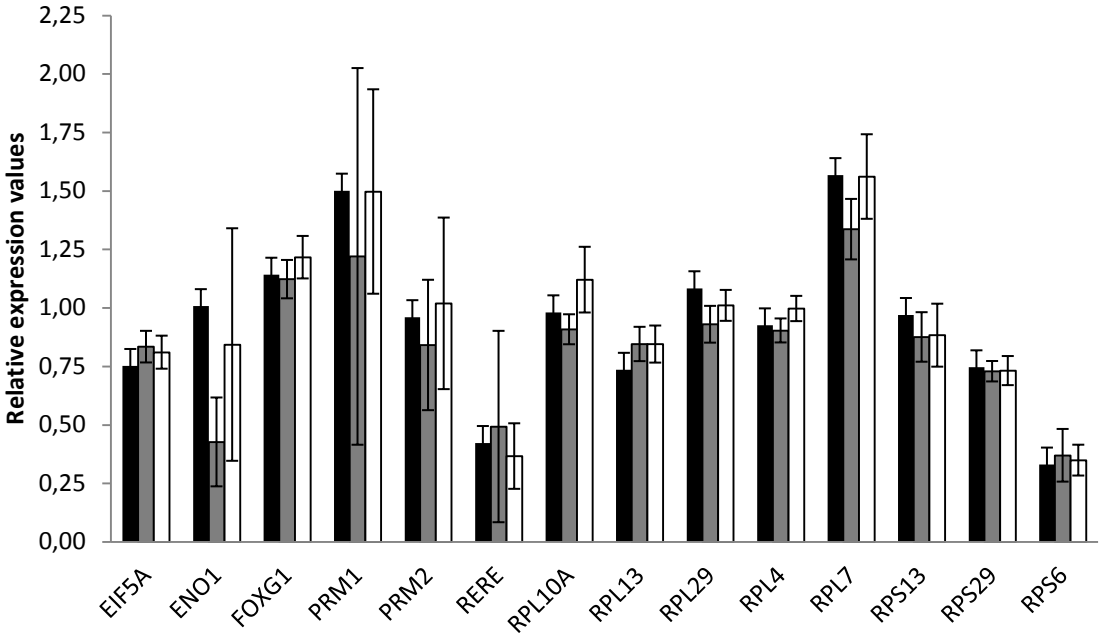


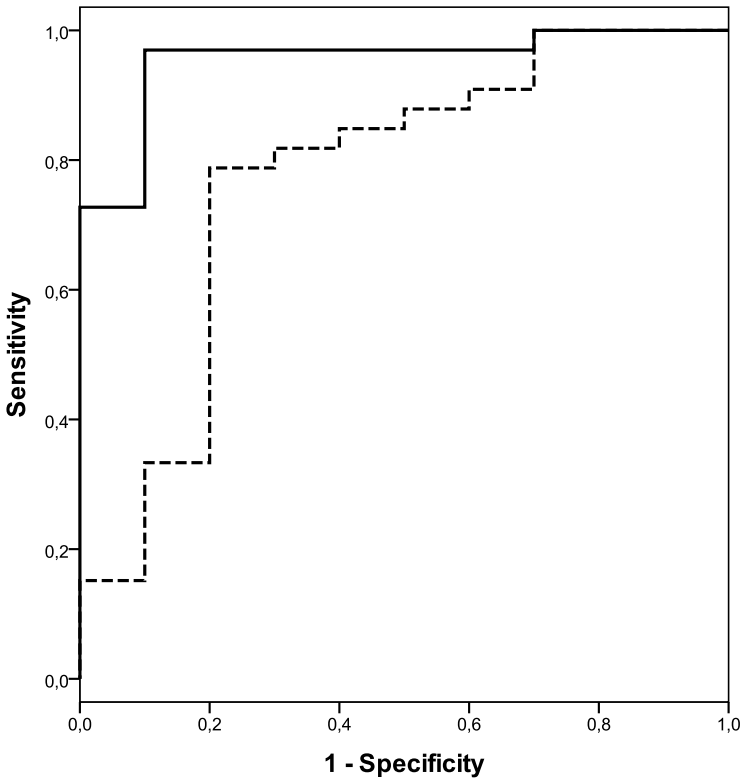
Figure 3

A.


Relative expression values
2,00
1,75
1,50
1,25
1,00
0,75
0,50
0,25
0,00
RBM9
RPL23A
RPS27
RPS27A
RPS3
RPS8
TOMM7
RPS18

B.


Relative expression values
2,25
2,00
1,75
1,50
1,25
1,00
0,75
0,50
0,25
0,00
EIF5A
ENO1
FOXG1
PRM1
PRM2
RERE
RPL10A
RPL13
RPL29
RPL4
RPL7
RPS13
RPS29
RPS6


Sensitivity
1 - Specificity
0,0
0,2
0,4
0,6
0,8
1,0

Supplemental Table I. List of genes included on the TLDA1

Gene symbol	Gene name	Applied Assay ID	Ct value range	Role
PRM-1+	Protamine-1	Hs00358158_g1 [1]	24- 29	Nuclear condensation
PRM-2+	Protamine-2	Hs00172518_m1 [1]	22.5- 29	Nuclear condensation
RERE+	Arginine-glutamic acid dipeptide (RE) repeats	Hs00201558_m1 [1]	28- 32	Transcriptional regulation
FOXG1/FOXG1B +	Fork head box G1B	Hs01850784_s1 [2]	26- 28	Transcription regulation. (Early embryo patterning)
TEAD1/TEF1	TEA domain family member 1 (SV40 transcriptional enhancer factor) Trancriptional enhancer factor TEF-1	Hs00744253_s1 [2]	27- 30	Transcription regulation. (Early embryo patterning)
RBM9+	RNA binding motif protein 9	Hs00329214_s1 [2]	26- 29	Transcriptional regulation (RNA binding related)
LRRFIP1	Leucine rich repeat interacting protein 1	Hs00190993_m1 [1]	>33	Transcriptional regulation (RNA binding related)
IREB2	Iron-responsive element binding protein 2	Hs00386293_m1 [1]	31- >33	Transcriptional and translational regulation (RNA and protein binding related)
EIF3G	Eukaryotic translation initiation factor 3 delta subunit	Hs00186772_m1 [1]	31- >33	Translational regulation, initiation
EIF3J/EIF3SI	Eukaryotic translation initiation factor 3, subunit J (subunit I)	Hs00825842_g1 [1]	28.5- 32	Translational regulation, initiation
EIF3M/GA17	Eukaryotic translation initiation factor 3, subunit M	Hs00272235_m1 [1]	31- >33	Translational regulation, initiation. Fusogenic protein (Sperm-oocyte interaction?)
EIF5A+	Eukaryotic translation initiation factor 5A	Hs00744729_s1 [2]	26- 28	Translational regulation, initiation
EIF5	Eukaryotic translation initiation factor 5	Hs00820472_m1 [1]	31- >33	Translational regulation, initiation
RPS3+	Ribosomal protein S3	Hs02385124_g1 [1]	27- 30	Protein biosynthesis(ribosomal protein). DNA repair
RPS6+	Ribosomal protein S6	Hs02339423_g1 [1]	26- 29	Protein biosynthesis (ribosomal protein)
RPS8+	Ribosomal protein S8	Hs01374307_g1 [1]	25- 27	Protein biosynthesis (ribosomal protein)
RPS13+	Ribosomal protein S13	Hs01945436_u1 [3]	26- 28	Protein biosynthesis (ribosomal protein)
RPS16	Ribosomal protein S16	Hs01598518_gH [1]	27.5- 33	Protein biosynthesis (ribosomal protein)
RPS17#	Ribosomal protein S17	Hs00734303_g1 [1]	26- 27	Protein biosynthesis (ribosomal protein)
RPS18+	Ribosomal protein S18	Hs02387368_g1 [1]	24.5- 26.5	Protein biosynthesis (ribosomal protein)
RPS26	Ribosomal protein S26	Hs00955682_g1 [1]	30- >33	Protein biosynthesis (ribosomal protein)
RPS27+	Ribosomal protein S27	Hs01378332_g1 [1]	23- 25	Protein biosynthesis (ribosomal protein)
RPS27A / S27a+	Ribosomal protein S27a	Hs01923841_uH [3]	26.5- 29	Protein biosynthesis (ribosomal protein)
RPS29+	Ribosomal protein S29	Hs03004310_g1 [1]	23- 25	Protein biosynthesis (ribosomal protein)
RPL4+	Ribosomal protein L4	Hs03044647_g1 [1]	26- 28	Protein biosynthesis (ribosomal protein)
RPL5	Ribosomal protein L5	Hs00851991_u1 [1]	31- 33	Protein biosynthesis (ribosomal protein)
RPL7+	Ribosomal protein L7	Hs02596927_g1 [1]	24- 26	Protein biosynthesis (ribosomal protein)
RPL10A+	Ribosomal protein L10a	Hs01912344_uH [3]	27.5- 30	Protein biosynthesis (ribosomal protein)
RPL13+	Ribosomal protein L13	Hs00761672_s1 [2]	26- 29	Protein biosynthesis (ribosomal protein)
RPL17	Ribosomal protein L17, transcript variant 2	Hs01597859_m1 [1]	31- >33	Protein biosynthesis (ribosomal protein)
RPL17	Ribosomal protein L17, transcript variant 1	Hs00748900_s1 [2]	28- 31	Protein biosynthesis (ribosomal protein)
RPL23A+	Ribosomal protein L23a	Hs01921329_g1 [2]	27- 29	Protein biosynthesis (ribosomal protein)
RPL24	Ribosomal protein L24	Hs02338570_gH [1]	26- 30	Protein biosynthesis (ribosomal protein)
RPL27A	Ribosomal protein L27a	Hs00741143_s1 [2]	26- 29	Protein biosynthesis (ribosomal protein)
RPL29#	Ribosomal protein L29	Hs00988959_gH [2]	23- 25	Protein biosynthesis (ribosomal protein)
RPL30	Ribosomal protein L30	Hs00265497_m1 [1]	30- >33	Protein biosynthesis (ribosomal protein)
RPL35	Ribosomal protein L35	Hs00855441_gH [2]	25- 29	Protein biosynthesis (ribosomal protein)
RPLP2	Ribosomal protein, large, P2	Hs01115130_g1 [1]	26- 30	Protein biosynthesis (ribosomal protein)
FAU	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	Hs00609872_g1 [1]	27- 29	Protein biosynthesis (ribosomal protein)
RSL1D1	Ribosomal L1 domain containing 1	Hs00378363_g1 [1]	>33	Protein biosynthesis (ribosomal protein)
EEF2	Eukaryotic translation elongation factor 2	Hs01012839_g1 [1]	30- 33	Protein biosynthesis (ribosomal protein)
RPS4Y1	40S ribosomal protein S4, Y isoform (Y-linked 1)	Hs00606158_m1 [1]	32- >33	Protein biosynthesis (ribosomal protein)
MRPL40	Mitochondrial ribosomal protein L40	Hs00186843_m1 [1]	32- >33	Protein biosynthesis (mitochondrial ribosomal protein)
MRPS18B	Mitochondrial ribosomal protein S18B	Hs00204096_m1 [1]	32- >33	Protein biosynthesis (mitochondrial ribosomal protein)
FARSB	Phenylalanyl-tRNA synthetase beta chain cytoplasmic	Hs00271714_m1 [1]	32- >33	Protein biosynthesis (cytoplasmic protein)
COPS5	COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis)	Hs00272789_m1 [1]	32- >33	Protein biosynthesis (nuclear protein)
RPS6KA2	Ribosomal protein S6 kinase	Hs00179731_m1 [1]	31- >33	Protein amino acid modification (phosphorylation)
ST6GALNAC4	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 4	Hs00205241_m1 [1]	>33	Protein amino acid modification (glycosilation in Golgi apparatus)
NARS	Asparaginyl-tRNA synthetase	Hs00189846_m1 [1]	>33	Protein binding related (aspartyl-tRNA aminoacylation)
QARS	Glutaminyl-tRNA synthetase	Hs00192530_m1 [1]	32- >33	Protein binding related (glutaminyl-tRNA aminoacylation)
UBC	Ubiquitin C	Hs00824723_m1 [1]	29- 33	Protein amino acid modification (Ubiquitin conjugating system)
TMED2/RNP24	Transmembrane emp24 domain trafficking protein 2 / Homo sapiens coated vesicle membrane protein	Hs00607277_m1 [1]	>33	Protein transportation (cytoplasm protein)
SCAMP1	Secretory carrier membrane protein 1	Hs00792736_m1 [1]	>33	Protein transportation (cytoplasm protein)
VTI1B	Vesicle transport through interaction with t-SNAREs homolog 1B (yeast)	Hs00762282_s1 [2]	26- 30	Protein transportation (cytoplasm protein)
SLC29A2	Solute carrier family 29 (nucleoside transporters), member 2	Hs00155426_m1 [1]	>33	Protein transportation (cytoplasm protein)

SLC25A19	Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19, variant 2	Hs00222265_m1 [1]	>33	Protein transportation (mytochondrial protein)
SLC25A25	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25	Hs01595832_m1 [1]	>33	Protein transportation (mytochondrial protein)
SLC25A39	Solute carrier family 25, member 39	Hs00255405_m1 [1]	29.5- >33	Protein transportation (mytochondrial protein)
TOMM7+	Translocase of outer mitochondrial membrane 7 homolog (yeast)	Hs01628668_s1 [2]	28- 31	Protein transportation (mytochondrial protein)
SFXN3	Sideroflexin 3	Hs00229616_m1 [1]	>33	Protein transportation (mytochondrial protein)
CSE1L	CSE1 chromosome segregation 1-like (yeast)	Hs00354853_m1 [1]	31- >33	Protein-nucleus import (nuclear pore complex)
IPO5	Importin 5	Hs00267008_m1 [1]	32- >33	Protein-nucleus import (nuclear pore complex)
XPO1	Exportin 1 (CRM1 homolog, yeast)	Hs00185645_m1 [1]	32- >33	Protein-nucleus import (nuclear pore complex)
XPO7	Exportin 7	Hs00209262_m1 [1]	32- >33	Protein-nucleus import (nuclear pore complex)
KPNA2	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	Hs00818252_g1 [1]	32- >33	Protein-nucleus import (nuclear pore complex)
RANBP2	RAN binding protein 2	Hs00397898_g1 [1]	30- >33	Protein-nucleus import (nuclear pore complex)
PDIA3	Protein disulfide isomerase family A, member 3	Hs00607126_m1 [1]	32- >33	Protein folding (endoplasmic reticulum)
WBSCR21/ABHD11	Abhydrolase domain containing 11	Hs00541033_g1 [1]	>33	Catalytic activity (metabolic feature of spermatozoa?)
RNF144B/ IBRDC2	Ring finger 144B / IBR damin containing 2	Hs00403456_m1 [1]	31- >33	Catalytic activity, protein ubiquitination (metabolic feature of spermatozoa?)
CCNB1IP1	Cyclin B1 interacting protein 1	Hs00820463_g1 [1]	28- 32	Catalytic activity (metabolic feature of spermatozoa?)
ENO1+	Enolase 1 (alpha)	Hs00361415_m1 [1]	27- 33	Catalytic activity (metabolic feature of spermatozoa?)
COX5B	Cytochrome c oxidase subunit Vb	Hs00426948_m1 [1]	32- >33	Catalytic activity (metabolic feature of spermatozoa?)
FADS1	Fatty acid desaturase 1	Hs00203685_m1 [1]	>33	Catalytic activity (metabolic feature of spermatozoa?)
AKAP-4	A kinase (PRKA) anchor protein 4	Hs00275849_m1 [1]	30- 33	Signal transduction. Involved in sperm motility (fertilization and activation of oocyte)
FGFR1	Fibroblast growth factor receptor 1	Hs00241111_m1 [1]	>33	Signal transduction (spermatogenesis and fertilization?)
TM4SF6	Tetraspanin 6	Hs00170288_m1 [1]	32- >33	Signal transduction (spermatogenesis and fertilization?)
GRIN2C	Glutamate receptor, ionotropic, N-methyl D-aspartate 2C	Hs01016626_m1 [1]	>33	Signal transduction (spermatogenesis and fertilization?)
IL6ST	Interleukin 6 signal transducer	Hs00174360_m1 [1]	32- >33	Signal transduction (spermatogenesis and fertilization?)
VAV2	Vav 2 oncogene	Hs00610104_m1 [1]	32- >33	Signal transduction (spermatogenesis and fertilization?)
WNT5A	Wingless-type MMTV integration site family, member 5A	Hs00998537_m1 [1]	>33	Signal transduction. Embryonic development, Cellular differentiation and morphometric patterning
HLA-E	Major histocompatibility complex, class 1, E	Hs00428366_m1 [1]	31- >33	Signal transduction. Immune response protein Signal transducer protein (spermatogenesis and fertilization?)
eNOS/NOS3	Endothelial nitric oxide synthase, nitric oxide synthase 3 (endothelial cell)	Hs00167166_m1 [1]	32- >33	Capacitation
nNOS/NOS1	Neuronal nitric oxide synthase, nitric oxide synthase 1 (neuronal)	Hs00167223_m1 [1]	>33	Capacitation
CLGN	Calmegin precursor variant 1	Hs00189073_m1 [1]	31- >33	Testis-specific endoplasmic reticulum chaperone protein. (Sperm-egg interaction, Fertilization)
CLU	Clusterin	Hs00156548_m1 [1]	29- 33	Protein binding. Cell-cell interations; cellular processes for embryo development
PTHLH	Parathyroid hormone-like hormone	Hs00174969_m1 [1]	>33	Regulation of gene expression. Hormonal activity.(Pregnancy)
CRHBP	Corticotropin releasing hormone binding protein	Hs00181810_m1 [1]	>33	Signal transducer protein. Hormonal activity. (Pregnancy)
HPRT	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	Hs99999909_m1 [1]	32- >33	
HMBS	Hydroxymethylbilane synthase	Hs00609297_m1 [1]	>33	
PPIA	Cyclophilin A, peptidylprolyl isomerase A	Hs99999904_m1 [3]	24- >33	
PGM1	Phosphoglucomutase 1	Hs00160062_m1 [1]	32- >33	
GUSB	Glucuronidase, beta	Hs99999908_m1 [1]	>33	
PGK1	Phosphoglycerate kinase 1	Hs99999906_m1 [3]	>33	
TBP	TATA box binding protein	Hs00427620_m1 [1]	32- >33	
KIAA0999/ L19	KIAA0999 protein	Hs00228549_m1 [1]	32- >33	

Gene symbol in bold and underlined depicts those genes that showed positive PCR-amplifications in all samples under the conditions described in Materials and Methods section.
Those genes included in TLDA2 are indicated with + (target genes) or # (reference genes) symbols

[1] The assay probe spans an exon junction and thus, the assay should not detect gDNA; [2] Both primers and probe map within a single exon and thus, the assay will detect gDNA; [3] The amplified product spans an exon junction and the probe and/or one/ both primers sit within one exon. This assay may detect gDNA.