Sperm proteomic changes associated to early embryo quality after ICSI

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STRUCTURED ABSTRACT (255 words)

Research Question: Do alterations of human sperm protein profile affect embryo quality?

Design: Sperm proteins from 27 infertile patients undergoing ICSI were extracted, afterward labeled using tandem mass tags (TMT), and finally analyzed by two-dimensional liquid chromatography coupled with tandem mass spectrometry (2D-LC-MS/MS) following strict criteria. Correlations between sperm proteins abundance and quality of the ICSI-derived embryos at day 2 were evaluated first in idiopathic infertile couples (n=16), at both protein and peptide levels. After, those correlations were validated in a subset of infertile patients with a known male or female factor (n=11).

Results: A total of 18 sperm proteins were correlated with embryo quality after ICSI. Around 40 % of these sperm proteins coincided with the differential proteins identified in similar published studies correlating sperm proteome with different *in vitro* assisted reproductive technologies (ART) outcomes. Remarkably, a high percentage of poor-quality ICSI-derived embryos was associated with the alteration of the chaperonin TCP-1 ring complex (TRiC), which plays an important role in the folding of many essential proteins. Additionally, several sperm proteins with a known function in embryogenesis such as RUBVL1 were also correlated with early embryo quality. Interestingly, the analysis at the peptide level revealed the association of some correlations with specific post-translational modifications and isoforms.

Conclusions: Our results support the hypothesis that sperm proteome play a role in early embryogenesis. Moreover, several sperm proteins have emerged as potential biomarkers to predict *in vitro* ART outcome, leading to the possibility to be further developed to improve the management of idiopathic infertility couples.

KEYWORDS: Idiopathic infertility, Sperm proteome, Embryo quality, ICSI

KEY MESSAGE:

Sperm proteins seem to play a role in the correct development of the preimplantation embryo such as proteins of TRiC complex required for the correct folding of 10 % of eukaryotic proteins. Those sperm proteins could be further developed as relevant clinical biomarkers to the better counseling of idiopathic infertile couples.

INTRODUCTION

About 20 % of all couples in reproductive age are affected by infertility, which is a rising health problem with a direct impact on patient's quality of life (Skakkebaek et al., 2015). The male factor is estimated to be present in approximately half of the infertility cases (Krausz et al., 2015; Skakkebaek et al., 2015; Thonneau et al., 1991). Therefore, at least 7 % of men in reproductive age (15 to 60 years) are unable to achieve a live birth after twelve months of unprotected intercourse and often seek reproductive care (Skakkebaek et al., 2015).

Today, approximately 2.1 % of children born in Europe are conceived through assisted reproductive technologies (ART) (De Geyter et al., 2018). However, couples often have to face several attempts of ART cycles, becoming a costly and time-consuming process. Among the different *in vitro* fertilization (IVF) procedures available, the intracytoplasmic sperm injection (ICSI) is the one most widely used in the clinic. Specifically, it is used in almost half of the cases with a pregnancy success rate of 27 % per cycle (De Geyter et al., 2018). Although male and female factors contribute about equally to infertility (Krausz et al., 2015), the current clinical study of the infertile couples is mainly focused on female. The evaluation of men is just restricted to the examination of the semen parameters, which only reveals gross deficiencies in sperm count, motility, and morphology, but has a limited ability to predict the success of ART (World Health Organization, 2010). Nowadays, reproductive counseling for couples without any known female or male factor, currently diagnosed as unexplained or idiopathic infertility, represent a unique challenge, in particular for those idiopathic infertile couples who fail several *in vitro* ART cycles attempts. *A priori*, those unsuccessful *in vitro* ART cycles are attributed to alterations in the oocyte since traditionally it has been considered that the oocyte cytoplasm contains all the factors required for early embryogenesis (Swain and Pool, 2008). In contrast to this general view, the increasing use of high-throughput

technologies has revealed that, in fact, sperm provides DNA containing different epigenetic marks in combination with a complex population of proteins and RNAs that might be crucial for embryonic development (Carrell et al., 2015; Castillo et al., 2018; Hammoud et al., 2009; Jodar et al., 2013). These findings emphasize the need to develop alternative strategies to accurately evaluate male factors potentially involved in the first steps of embryogenesis.

A total of 6,871 non-redundant proteins have so far been identified by high-throughput proteomic strategies in human sperm (Amaral et al., 2014a, 2013; Baker et al., 2013; Castillo et al., 2018; de Mateo et al., 2011; Martínez-Heredia et al., 2006; Wang et al., 2013). Although most of these proteins are related to spermatogenesis and sperm functionality (Amaral et al., 2014a, 2014b; Castillo et al., 2018), 11 % of sperm proteins seem to be involved in fertilization, early embryo development or regulation of gene expression after embryonic genome activation (Castillo et al., 2018). Supporting this hypothesis, several studies have found strong correlations between alterations in sperm proteomic profile and low fertilization rates, blastocyst quality, as well as unsuccessful IVF or ICSI cycles (Azpiazu et al., 2014; Frapsauce et al., 2014; Légaré et al., 2014; Liu et al., 2018; McReynolds et al., 2014; Zhu et al., 2013). With the only exception of the study published by Zhu and colleagues in 2013, the rest of these comparative proteomic studies were performed using pools of samples categorized according to *in vitro* ART outcomes. It is assumed that samples are homogeneous once they are pooled, however, it is known that several variables including unknown male and female factors could result in low fertilization rates, low blastocyst quality or unsuccessful ART cycles. Therefore, the use of individual samples could help to detect sample-specific alterations contributing to an unsuccessful ART cycle. In the present work, we have determined changes on sperm protein profile correlating with ICSI-derived embryo quality at day 2 using a comparative TMT-2D-LC-MS/MS proteomic approach that enabled us the proteomic

study of individual samples at a reasonable cost. Our results gain insight into the role of specific sperm proteins in early embryogenesis, which could be further developed as potential clinical biomarkers to predict the success of *in vitro* ART.

MATERIALS AND METHODS

Sample collection and embryo quality assessment

Human semen samples were obtained from patients undergoing ICSI at the Assisted Reproduction Unit of the Hospital Clínic of Barcelona (FIV Clínic-ICGON, Spain). All patients signed the corresponding informed consent forms and all human material was used in accordance with the appropriate ethical and Internal Review Board guidelines of the Hospital Clínic of Barcelona (Spain). The surpluses of semen samples prepared for *in vitro* ART from 27 infertile couples were used in this study (Table 1).

Semen samples were collected by masturbation into a sterile container after 2-7 days of sexual abstinence. After liquefaction, routine semen analyses were performed by following the World Health Organization (WHO) guidelines (World Health Organization, 2010). Patients were classified according to seminal parameters and phenotypic traits as: (i) idiopathic infertile couples (n=16); (ii) infertile couples with a male factor according to their seminal parameters (n=4), including asthenozoospermic patients (< 32 % sperm progressive motility) or oligoasthenozoospermic patients (< 39 million of total sperm and < 32 % sperm progressive motility); and (iii) infertile couples with a known female factor, including women over 40 years old and with obesity (n=7; Table 1).

Spermatozoa were purified from seminal plasma through a discontinuous density gradient (90 %, 70 %, 50 % PureSperm, NidaCon Laboratories AB, Gothenburg, Sweden) by centrifugation at 400 g for 20 min. Carefully, pellets were collected and washed in All grad Wash® (The LifeGlobal® Group, Connecticut, USA) by

centrifugation at 400 g for 10 min. Afterward, pellets were prepared by the routine swim-up procedure. Briefly, sperm pellets were gently covered with 0.5 ml of global® total® for Fertilization medium (The LifeGlobal® Group) and incubated for 20 min at 37 °C in 7 % of CO_2 and 7 % of O_2. After picking up spermatozoa for the ICSI procedure, the surplus of sperm cells was assessed for sperm count and motility using a Makler counting chamber (Selfi medical instruments LTD, Haifa, Israel) and for sperm vitality using 0.5 % (w/v) Eosin Y, by following the WHO guidelines (World Health Organization, 2010). The remaining material was cryopreserved in liquid nitrogen using CryoSperm (Origio, Malov, Denmark) in accordance with the manufacturer's instructions until the sample was processed for proteomics analysis (Figure 1).

Embryo assessment data (n=150) was collected after the ICSI procedure in accordance with the standard assisted reproduction clinic protocol. Briefly, fertilized oocytes were examined and only those with two pronuclei were further cultured in 20 µl drops of global® culture media (The LifeGlobal® Group) under mineral oil. Embryos at day 2 (D2; 43-45h after ICSI) were scored based on the morphological features according to the embryo assessment criteria established by the Spanish Society, *Asociación Española para el Estudio de la Biología de la Reproducción* (ASEBIR) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Of Embryology, 2011). The embryo quality assessment was mainly based on six parameters: 1) the number of blastomeres, 2) symmetry of the blastomeres, 3) size and distribution of the embryo fragmentation, 4) presence of multi-nucleation 5) cytoplasmic anomalies such as the presence of vacuoles and 6) zona pellucida irregularities. ASEBIR consensus scheme scores D2 embryos as top quality (A), good-quality but not suitable for elective single-embryo transfer (B), impaired embryo quality (C) and not recommended for transfer (D) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Of Embryology, 2011). The percentages

of good-quality embryos (embryos graded as A or B) and poor-quality embryos (embryos graded as D) derived from an ICSI cycle were calculated for each couple included in the study (Table 1).

Protein purification and peptide tandem mass tag (TMT)-labeling

Sperm proteins were solubilized by incubation of spermatozoa during 1 h at room temperature with 1 % sodium dodecyl sulfate (SDS) in 100 mM triethylammonium bicarbonate (TEAB). Cell lysates were centrifuged at 17,500 g for 10 min at 4 ºC and the supernatants containing the soluble protein fraction were quantified utilizing Pierce™ Reducing Agent Compatible BCA Protein Assay Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. From each sample, 30 µg of proteins were used for TMT-10 plex labeling by following the manufacturer's instructions with minor modifications, as described elsewhere (Bogle et al., 2017). Before peptide labeling, equivalent aliquots of each individual sample (n=27) were merged in a single internal control. Subsequently, each sample and the internal control were individually labeled with TMT reporter ions with intensities from m/z 126 to m/z 131: TMT-126 was used for the internal control and -127N, -127C -128N, -128C, -129N, -129C, -130N, -130C, -131 for individual samples (Supplementary figure 1). To quench the reaction, each tube was incubated with 2.6 µL of 8 % hydroxylamine for 15 min. After quenching, TMT-labeled samples were combined at equal volume in three different pools (A, B and C) with nine samples and the internal control each (Supplementary figure 1). The pooled samples were dried in a speed-vacuum and, subsequently, 20 µg of peptides from each pool were cleaned-up with Pierce C18 Spin Columns (Thermo Scientific), in accordance with the manufacturer's instructions.

2D-LC-MS/MS analysis

A total of 10 µg of TMT-labeled peptides from each pool (A, B and C) were fractioned using a Zorbax 300 HILIC column (1.8 µm, 2.1 x 50 mm, 300 Å; Agilent Technologies, Santa Clara, USA) coupled to a Surveyor MS Pump (Thermo Finnigan, Waltham, USA). Two different buffers were employed for the fractionation: Buffer A (97 % acetonitrile (ACN), 3 % H2O) and Buffer B (97 % 50 mM ammonium acetate pH = 4.5, 3 % ACN). A gradient of 15 min was run from 5 % to 100 % buffer B and twenty fractions were collected from each pool. Each fraction was dried under a speed vacuum, reconstituted and loaded to a PepMap 100 trap column (300 µm x 5 mm, 5 µm, 100 Å, Thermo Fisher Scientific) to clean and pre-concentrate the fractions. For the liquid chromatography, a PepMap C18 column (75 µm × 15 cm, 3 µm, 100 Å, Thermo Fisher Scientific) was employed with a 120 min linear gradient with buffer A (97 % H_2O, 3 % ACN, 0.1 % formic acid (FA)) and buffer B (97 % ACN, 3 % H_2O, 0.1 % FA) as follows: 0-4 min, 0 % buffer B; 4-100 min, 32.5 % buffer B; and 100-120 min, 100 % buffer B. The MS/MS analysis was performed by a nano-LC ultra 2D eksigent (AB Sciex, Madrid, Spain) attached to an LTQ-Orbitrap Velos coupled to a nanospray ion source (Thermo Fisher Scientific). The LTQ Orbitrap Velos (Thermo Fisher Scientific) settings included 30,000 of resolution for the MS1 scans at 400 m/z for precursor ions followed by 30,000 of resolution for the MS2 scans of the 20 most intense precursor ions at 400 m/z, in positive ion mode. The mass range was set 300-1,600 m/z. The acquisition of MS/MS data was performed using Xcalibur 2.2 (Thermo Fisher Scientific). Normalized collision energy for HCD-MS2 was set to 42 %.

Protein identification and quantification

MS/MS data output was analyzed by Proteome Discoverer 1.4. (ThermoFisher Scientific). For database searching, processed data was submitted to the in-house Homo sapiens UniProtKB/Swiss-Prot (last modified February 2014; 20,240 protein entries) using SEQUEST version 28.0 (Thermo Fisher Scientific). Percolator search node was also applied. Searches were performed using the following settings: 2

maximum missed cleavages for trypsin; TMT-labeled in N-terminus, lysine, histidine, serine, tyrosine (+229.163 Da), and methionine oxidation (+15.995 Da) as dynamic modifications, and cysteine carbamidomethylation (+57.021 Da) as a static modification; 20 ppm precursor mass tolerance, 0.05 Da fragment mass tolerance, 5 mmu peak integration tolerance, and most confident centroid peak integration method.

Criteria used to accept protein identification included a minimum of 1 unique peptide matched per protein, with a 1 % false discovery rate (FDR). The “enable protein grouping” option was disabled to avoid possible ambiguity in the identification of different isoforms of the same proteins during quantification. For protein quantification data, normalized TMT quantitative values expressed as the ratio of the intensities of reporter ions from HCD MS2 spectra from each individual sample (TMT-127 to TMT-131) with the internal control (TMT-126) were obtained by Proteome Discoverer software from each identified spectrum, as previously described (Barrachina et al., 2019). Only those peptides quantified by ≥ 2 peptide-spectrum matches (PSMs) in all the samples with a coefficient of variation < 50 % in at least 75 % of the samples were considered for further statistical analyses. Significant statistical differences between groups were evaluated after the normalization of the relative proteomic quantification values by $\log_2$ transformation (Barrachina et al., 2019).

Statistical analyses

All statistical analyses were performed using R software version 3.4.4 (http://www.r-project.org) or GraphPad Prism software version 7.01 (GraphPad Software Inc., San Diego, USA). Multiple Pearson correlation analyses between $\log_2$ transformed values of relative proteomic quantification at both protein and peptide levels with either % A+B or % D embryos were separately conducted for the 3 groups, (i) idiopathic infertile couples, (ii) infertile couples with a male factor and (iii) infertile couples with a female

factor. P-values < 0.05 were considered statistically significant. Additionally, UniProt Knowledgebase (https://www.uniprot.org/) was employed to identify possible protein isoforms and post-translational modifications.

Gene ontology enrichment analysis was carried out using Gene Ontology Consortium database (http://www.geneontology.org/) based on PANTHER v. 14 database (Released on 2019-01-01) in order to predict alterations in specific cellular pathways. The significance of the enrichment was calculated by Fisher's exact test. P-values < 0.05 after Bonferroni adjustment were considered statistically significant.

RESULTS

Identification and reliable quantification of sperm proteins

The combined 2D-LC-MS/MS proteomic analysis of the 27 sperm samples included in this study resulted in the identification of 1,409 proteins, from which 727 were detected in all runs (Supplementary Table 1). After applying our strict quantification criteria (at least one unique peptide quantified with ≥ 2 PSMs in all samples and with a coefficient of variation < 50 % in at least 75 % of the samples), 511 peptides comprised in 210 proteins were selected for further correlation analyses (Figure 1 and Supplementary Table 2). Of note, this subset of proteins is functionally enriched in proteins involved in energy production, spermatogenesis, sperm motility, fertilization and telomere maintenance (Supplementary Table 3). Complementarily, the use of the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org) allowed to identify 3 proteins whose depletion induces embryonic lethality before implantation in mouse, the 40S ribosomal protein SA (RPSA), the Outer dense fiber protein 2 (ODF2) and the RuvB-like 1 (RUVBL1)), suggestive of their critical role in early embryogenesis.

Correlation of sperm proteins from idiopathic infertile couples with embryo quality at day 2 after ICSI

Analyzing the correlation of the relative abundance of sperm proteins from the subgroup of idiopathic infertile couples (n=16), two sperm proteins, sperm equatorial segment protein 1 (SPESP1) and four and a half LIM domains protein 1 (FHL1), displayed significantly positive and negative correlations with the percentage of ICSI-derived good-quality embryos, respectively (Figure 2; $p < 0.05$). Additionally, 16 sperm proteins were significantly correlated with the percentage of ICSI-derived poor quality embryos (Table 2; $p< 0.05$). According to the Gene Ontology Consortium Database, those 16 proteins are functionally enriched in processes related to telomere maintenance, supporting the importance of telomeres during early embryo development (Turner et al., 2010; Supplementary Table 4).

Correlations between sperm proteins at the peptide level from idiopathic infertile couples and embryo quality

Up to 7 of the 18 sperm proteins correlated with embryo quality were reliably quantified with more than 1 peptide. Specifically, these proteins are the protein T-complex protein 1 subunit gamma (CCT3), the protein T-complex protein 1 subunit theta (CCT8), the enolase 1 (ENO1), the zona pellucida binding protein (ZPBP), the mitochondrial protein Peroxiredoxin-5 (PRDX5), RUVBL1 and SPESP1 (Table 3). Pearson correlation analysis at the peptide level showed that the totality of quantified peptides for SPESP1 (3 peptides) and RUVBL1 (2 peptides) were significantly correlated ($p < 0.05$) or at least showed a tendency ($p < 0.1$) with the percentage of A+B and D embryos, respectively (Table 3, Figure 3A and Supplementary Figure 2). However, one or more peptides of the remaining five sperm proteins did not correlate with embryo quality (Table 3). This heterogeneity observed at the peptide level may be explained by the presence of either post-translational modifications (PTMs) or protein isoforms (Figure 3B-C and Supplementary Figure 2). For instance, only 2 out of 3 peptides quantified for CCT3 showed a strong correlation with poor-quality embryos. Interestingly, the non-correlated peptide contained a threonine (T) residue

that could be target of phosphorylation (Figure 3B). Similarly, only 1 out of 2 peptides quantified for the PRDX5 was correlated with the percentage of D embryos. However, the presence of different isoforms produced by alternative splicing could explain this observation. In this case, whereas the non-correlated peptide is common in all PRDX5 isoforms provided by UniProt Knowledgebase, the correlated peptide is exclusively absent in PRDX5 isoform 4. Therefore, these results suggest that all the PRDX5 isoforms present in sperm with the exception of the isoform 4 are correlated with embryo quality (Figure 3C).

Reliability of the identified correlations between sperm proteins and embryo quality: a comprehensive validation in a different subset of infertile patients as well as in independent studies

The 18 sperm proteins correlated with embryo quality in idiopathic infertile couples were assessed in a different subset of infertile patients with a known male or female factor (n=11) for its validation. While the positive significant correlation between the abundance of the uncharacterized protein C7orf61 and the percentage of D embryos was confirmed in infertile patients with known male factor ($p < 0.05$), no correlation was found when there is a known female factor as expected (Supplementary Table 5). It is also interesting to note that the subgroup of infertile patients with known male factor showed a tendency for the correlation between SPESP1 abundance and the percentage of good-quality embryos ($p = 0.057$) (Supplementary Table 5).

Additionally, some sperm proteins correlated with embryo quality in the current study have been also identified altered in similar published studies (Supplementary Table 6). This is exemplified by the strong association between blastocyst development and the differential abundance of Cysteine-Rich Secretory Protein 2 (CRISP2) and PRDX5 (McReynolds et al., 2014). Likewise, the abundance of FHL1, RUVBL1,

ENO1, C7orf61, and ZPBP in sperm was related to the pregnancy outcome through conventional IVF or ICSI (Azpiazu et al., 2014; Légaré et al., 2014; Liu et al., 2018).

DISCUSSION

Traditionally, the focus in reproductive medicine has been women while the evaluation of the male partner has generally been restricted to the evaluation of seminal parameters. This is reflected in the fact that, at present, there are not drugs either to be prescribed to males or to treat sperm *in vitro* in order to improve male fertility. In the same line, there is a lack of validated molecular tests based on the study of semen to predict the success of ARTs. The limited tools currently available for male infertility diagnosis, prognosis and therapy are mainly due to the incomplete understanding of the father's contribution to his progeny. Therefore, deciphering what molecular causes are involved in male infertility may shed new light on essential paternal components for embryogenesis and may promote the development of molecular tests that would finally aid the change of male fertility diagnosis paradigm (Jodar et al., 2015).

Comparative high-throughput proteomics of individual sperm samples is a good strategy to discover new physiological and pathological mechanisms of male fertility as well as clinically useful biomarkers (Jodar et al., 2017). However, in order to avoid false positive results, it is needed to establish strict criteria for both protein identification and quantification. In the present study, 18 sperm proteins have been correlated with ICSI-derived embryo quality. It is interesting to note that the alteration of 7 of those 18 sperm proteins has also been associated with *in vitro* ART outcomes in similar published studies (Supplementary Table 6). This concordance between our results and those studies published by others (around 40 %) is much higher than the observed when comparing other phenotypes. For instance, there is just a 9 % of concordance between comparative high-throughput sperm proteomics studies of asthenozoospermic patients (Jodar et al., 2017). This observation leads us to

conclude that applying our strict criteria for protein identification and quantification is a good approach to reduce false positive associations.

Only 2 sperm proteins were significantly correlated with the percentage of good-quality (A+B) ICSI-derived embryos in idiopathic infertile couples (Figure 2), the zinc finger protein FHL1 and SPESP1, which is essential for sperm-egg binding and fusion (Fujihara et al., 2010). Although the ICSI procedure overcomes the sperm-oocyte binding and fusion processes, it has been shown that *Spesp1* disruption in mouse causes an aberrant distribution of various sperm proteins, resulting in fertilized oocytes that do not yield high-quality embryos (Fujihara et al., 2010).

Even more interesting, 16 sperm proteins were correlated with the percentage of poor-quality ICSI-derived embryos (Table 2). Of note, 5 of those 16 differential sperm proteins (t-complex protein 1 subunit alpha (TCP1), t-complex protein 1 subunit eta (CCT7), t-complex protein 1 subunit beta (CCT2), CCT3, and CCT8)) are components of the 8-membered chaperonin-containing T-complex (TRiC). TRiC is essential for cell survival since it is responsible for the correct folding of 10 % of the eukaryotic proteome (Leitner et al., 2012). Among the proteins that require TRiC for a correct folding is found the telomerase cofactor telomerase Cajal body protein 1 (TCAB1), which is essential for the telomerase function (Freund et al., 2014). Therefore, the negative correlation among TRiC components abundance and the percentage of D embryos supports previous findings, which positively correlated the sperm telomere length with the quality of early embryonic development and the embryo morphology, but not with pregnancy rates (Torra-Massana et al., 2018; Yang et al., 2015). Other sperm proteins that were also negatively correlated with the percentage of poor-quality embryos in our analysis have been described previously as essential in early embryogenesis such as, RUBVL1, PRDX5 and peroxiredoxin-6 (PRDX6). One the one hand, RUBVL1 is a chromatin remodeler potentially involved in the proliferation of the inner cell mass and, for this reason, *Rubvl1*-null embryos do not reach the

blastocyst stage (Bereshchenko et al., 2012). On the other hand, PRDX5 and PRDX6 have essential antioxidant activity during early embryogenesis (Leyens et al., 2004). The possible role in the first steps of the embryogenesis of the remaining 8 proteins that were correlated with poor-quality embryos still needs to be elucidated. However, it is remarkable that four of them (CRISP2, C7orf61, ENO1, and ZPBP; Supplementary Table 6) have also been detected by others with an altered abundance according to *in vitro* ART outcomes (Azpiazu et al., 2014; Légaré et al., 2014; Liu et al., 2018).

The strong correlation of both SPESP1 and C7orf61 with embryo quality is remarkable since they are maintained in infertile patients with abnormal seminal parameters, while they are lost in infertile couples with a known female factor. These results validate the potential predictive value of the sperm proteins SPESP1 and C7orf61 to estimate the quality of the *in vitro* ART-derived embryos in infertile couples without any known female factor and regardless the presence of alterations in the seminal parameters. The validation of the predictive value of these sperm protein biomarkers in a higher number of infertile patients by targeted proteomics or other techniques, such as a western blot or ELISA, is now needed in order to establish its applicability into the clinical practice. However, PTMs and different isoforms of the target proteins should be also taken into consideration before to design the validation tests, according to our results derived from the proteomic analysis at the peptide level.

This high-throughput proteomic analysis of idiopathic infertile patients classified according to the quality of ICSI-derived embryos has thus provided some insights into the sperm contribution to the early embryogenesis, suggesting novel potentially useful biomarkers to be applied in the clinics. It is important to note that the negative correlation of several TRiC complex components with a poor embryo quality supports the hypothesis that sperm telomeres are important for correct embryogenesis. Moreover, several sperm proteins have emerged as potential candidates to predict

the success of *in vitro* ART technologies, especially SPESP1 and C7orf61 proteins, since they have been associated with embryo quality regardless any putative alteration on the seminogram. Altogether indicates that our results might be applicable to the clinical practice with the capacity to better diagnose the infertile couples and predicting the success of the ART.

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FIGURE LEGENDS

Figure 1. Study design. Overall procedure employed for the identification and quantification of proteins in the different types of patients through TMT-10plex peptide labeling followed by 2D-LC-MS/MS, and the computational pipeline.

Figure 2. Correlation between sperm protein abundance and percentage of good-quality embryos in idiopathic infertile patients. Scatter plots of normalized SPESP1 and FHL1 protein abundance generated by 2D-LC-MS/MS (log_2 intensity ratio of the proteins) and % good-quality embryos (A+B) derived from ICSI. The Pearson correlation coefficient (r) and its p-value (p) are indicated for both correlations.

Figure 3. Correlations of sperm proteins with embryo quality (% A+B or % D) at the peptide level. A) All the quantified peptides (3 peptides) of SPESP1 were correlated ($p < 0.05$) or at least showed a tendency ($p < 0.1$) with the percentage of A+B embryos. B) Two of the three quantified peptides of CCT3 were correlated with the percentage of D embryos. Interestingly, the non-correlated peptide could contain a post-translational modification in a specific residue as described in the Uniprot Knowledgebase. C) Only one peptide of PRDX5 was correlated with the percentage of D embryos, most probably due to PRDX5-isoforms sequence differences. All isoforms of PRDX5 contain the non-correlated peptide; in contrast, the correlated peptide is missing in a specific isoform of PRDX5 (Isoform 4). Peptides showing correlations with p-value < 0.05, < 0.1 and > 0.1 are indicated in green, light green and red, respectively. Underlined and enlarged amino acid residues indicate the absence of a part of the amino acid sequence due to an isoform or the presence of post-translationally modified residues.

Table 1. Sample characteristics showing semen parameters, phenotypic traits, and reproductive clinical information.

				ART data							
							Embryo quality at day 2				
Sample number	Male age (years)	Seminogram	Female age (years)	Recovered oocytes	MII Oocytes	Fertilization rate (%)	A + B (%)	C (%)	D (%)	Total embryos analyzed	Pregnancy achievement
Idiophatic infertility											
1	42	Normozoospermia	36	5	4	75,0	0,0	66,6	33,3	3	N
2	43	Normozoospermia	<35*	16	16	68,8	9,1	63,6	27,3	11	N
3	39	Normozoospermia	38	7	7	85,7	16,6	50,0	33,0	6	N
4	40	Normozoospermia	33	6	6	100,0	16,6	83,3	0,0	6	N
5	38	Normozoospermia	39	8	8	62,5	20,0	20,0	60,0	5	N
6	34	Normozoospermia	34	7	7	42,9	33,3	66,6	0,0	3	N
7	37	Normozoospermia	37	9	7	85,7	50,0	33,3	16,6	6	N
8	39	Normozoospermia	37	8	7	100,0	57,0	28,5	14,2	7	Y
9	37	Normozoospermia	36	8	6	100,0	66,6	16,6	16,6	6	Y
10	37	Normozoospermia	32	10	8	75,0	66,6	16,6	16,6	6	N
11	37	Normozoospermia	38	8	6	50,0	66,6	33,3	0,0	3	Y
12	32	Normozoospermia	30	5	5	60,0	66,6	33,3	0,0	3	N
13	39	Normozoospermia	<35*	9	7	100,0	71,4	14,3	14,3	7	Y
14	38	Normozoospermia	39	8	8	75,0	83,3	16,6	0,0	6	Y
15	37	Normozoospermia	37	5	4	75,0	100,0	0,0	0,0	3	Y
16	36	Normozoospermia	36	8	7	100,0	100,0	0,0	0,0	7	Y
Male factor											
1	36	Oligoasthenozoospermia	34	9	6	83,3	20,0	80,0	0,0	5	Y
2	38	Oligoasthenozoospermia	36	16	13	61,5	42,0	14,0	42,0	7	N
3	39	Asthenozoospermia	39	15	7	85,7	66,6	16,7	16,7	6	Y
4	39	Asthenozoospermia	38	17	13	84,6	81,8	18,1	0,0	11	N
Female factor											
1	44	Normozoospermia	41	7	5	60,0	33,3	33,3	33,3	3	N
2	36	Normozoospermia	45	6	5	83,3	40,0	20,0	40,0	5	N
3	39	Normozoospermia	41	10	9	66,7	66,6	16,7	16,7	6	N
4	39	Normozoospermia	39	14	6	100,0	66,7	33,3	0,0	6	N
5	40	Normozoospermia	40	5	5	80,0	75,0	25,0	0,0	4	Y
6	42	Asthenozoospermia	42	8	7	85,7	100,0	0,0	0,0	6	N
7	40	Normozoospermia	40	12	9	33,3	100,0	0,0	0,0	3	Y

*Oocyte donation; N: no; Y: yes

Table 2. Pearson correlation analysis between normalized relative sperm protein abundance (2D-LC-MS/MS data) and percentage of poor-quality embryos (D). Pearson correlation coefficient and p-value are indicated in the table.

Gene name	UniprotKB	Pearson correlation coefficient (*r*-value)	Significance (*p*-value)
CCT7	Q99832	-0,646	0,007
CCT2	P78371	-0,641	0,007
CCT3	P49368	-0,626	0,010
CCT8	P50990	-0,622	0,010
ENO1	P06733	-0,587	0,017
CA2	P00918	-0,580	0,019
PRDX6	P30041	-0,560	0,024
RUVBL1	Q9Y265	-0,547	0,028
TCP1	P17987	-0,544	0,030
CRISP2	P16562	-0,541	0,030
ZPBP	Q9BS86	-0,520	0,039
GLB1L	Q6UWU2	-0,516	0,041
DPCD	Q9BVM2	-0,510	0,043
PSMA6	P60900	-0,507	0,045
C7orf61	Q8IZ16	0,502	0,048
PRDX5	P30044	-0,499	0,049

Table 3. Pearson correlation analysis between embryo quality (% A+B embryos or % D embryos) and normalized relative sperm peptide abundance (2D-LC-MS/MS data) for the 7 sperm proteins identified as correlated at protein level and quantified with more than two peptides. Pearson correlation coefficient (r-value) and p-value are provided.

% A + B				
UniprotKB	**Gene Name**	**Peptide sequence and modifications**	**Pearson correlation coefficient (r-value)**	**p-value**
Q6UW49	***SPESP1***	aATVFNTLk N-Term(TMT10Plex); K9(TMT10Plex)	0,5661	**0,0222**
		sPVTTLDk S1(TMT10Plex); K8(TMT10Plex)	0,4769	*0,0618*
		sQLLPVGR S1(TMT10Plex)	0,5014	**0,0478**
% D				
Protein	**Gene Name**	**Peptide sequence and modifications**	**Pearson correlation coefficient (r-value)**	**p-value**
P49368	***CCT3***	iPGGIIEDScVLR N-Term(TMT10Plex); C10(Carbamidomethyl)	-0,5017	**0,0477**
		tLIQNcGASTIR N-Term(TMT10Plex); C6(Carbamidomethyl)	-0,3572	0,1745
		vQSGNINAAk N-Term(TMT10Plex); K10(TMT10Plex)	-0,6326	**0,0085**
P50990	***CCT8***	aLAENSGVk N-Term(TMT10Plex); K9(TMT10Plex)	-0,6596	**0,0054**
		aVDDGVNTFk N-Term(TMT10Plex); K10(TMT10Plex)	-0,6368	**0,008**
		lATNAAVTVLR N-Term(TMT10Plex)	-0,3358	0,2035
P06733	***ENO1***	eGLELLk N-Term(TMT10Plex); K7(TMT10Plex)	-0,5593	**0,0243**
		gNPTVEVDLFTSk N-Term(TMT10Plex); K13(TMT10Plex)	-0,6453	**0,0069**
		lMIEMDGTENk N-Term(TMT10Plex); K11(TMT10Plex)	-0,4831	*0,058*
		lNVTEQEk N-Term(TMT10Plex); K8(TMT10Plex)	-0,4129	0,1119
		tIAPALVSk N-Term(TMT10Plex); K9(TMT10Plex)	-0,1103	0,6842
		yISPDQLADLYk N-Term(TMT10Plex); K12(TMT10Plex)	-0,5463	**0,0286**
Q9Y265	***RUVBL1***	aVLLAGPPGTGk N-Term(TMT10Plex); K12(TMT10Plex)	-0,4906	*0,0537*
		lDPSIFESLQk N-Term(TMT10Plex); K11(TMT10Plex)	-0,5013	**0,0479**
Q9BS86	***ZPBP***	aYVMLHQk N-Term(TMT10Plex); K8(TMT10Plex)	-0,4429	*0,0858*
		fFNQQVEILGR N-Term(TMT10Plex)	-0,1008	0,7103
		iVGSTSFPVk N-Term(TMT10Plex); K10(TMT10Plex)	-0,52	**0,039**
		nAELIDPSFQWYGPk N-Term(TMT10Plex); K15(TMT10Plex)	-0,5808	**0,0183**
P30044	***PRDX5***	lLADPTGAFGk N-Term(TMT10Plex); K11(TMT10Plex)	0,04154	0,8786
		vNLAELFk N-Term(TMT10Plex); K8(TMT10Plex)	-0,6012	**0,0138**

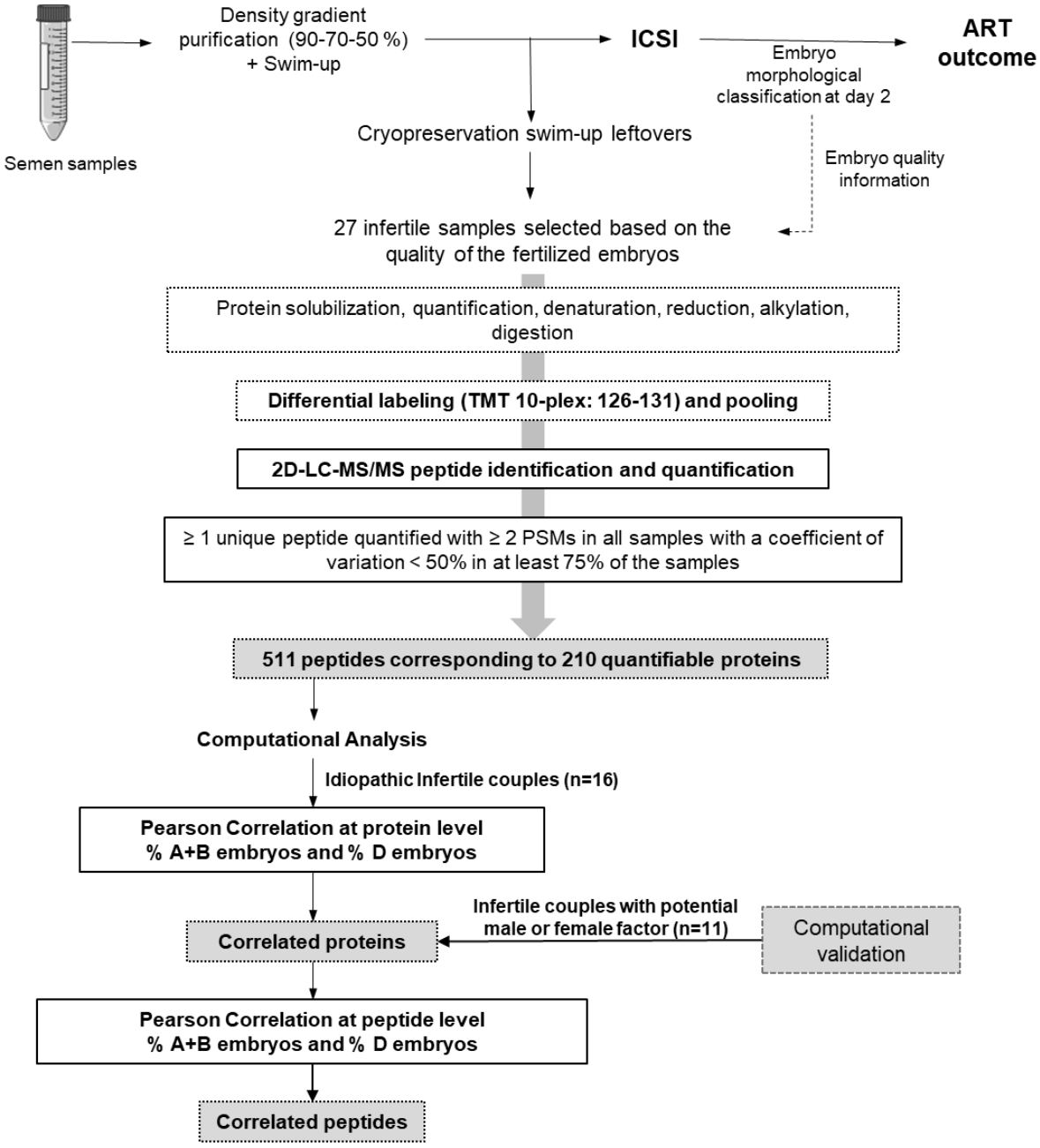
Semen samples
Density gradient purification (90-70-50 %) + Swim-up
ICSI
Embryo morphological classification at day 2
ART outcome
Cryopreservation swim-up leftovers
Embryo quality information
27 infertile samples selected based on the quality of the fertilized embryos
Protein solubilization, quantification, denaturation, reduction, alkylation, digestion
Differential labeling (TMT 10-plex: 126-131) and pooling
2D-LC-MS/MS peptide identification and quantification
≥ 1 unique peptide quantified with ≥ 2 PSMs in all samples with a coefficient of variation < 50% in at least 75% of the samples
511 peptides corresponding to 210 quantifiable proteins
Computational Analysis
Idiopathic Infertile couples (n=16)
Pearson Correlation at protein level % A+B embryos and % D embryos
Infertile couples with potential male or female factor (n=11)
Computational validation
Correlated proteins
Pearson Correlation at peptide level % A+B embryos and % D embryos
Correlated peptides

Figure 1

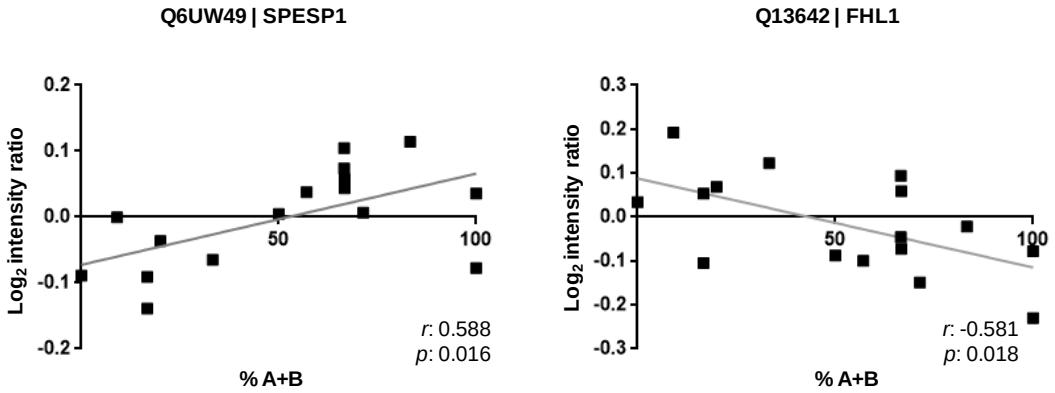
Q6UW49 | SPESP1
Log2 intensity ratio
0.2
0.1
0.0
-0.1
-0.2
50
100
r: 0.588
p: 0.016
% A+B
Q13642 | FHL1
Log2 intensity ratio
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
50
100
r: -0.581
p: 0.018
% A+B

Figure 2

A) Correlation of SPESP1 at peptide level with % A+B embryos.

MKPLVLLVALLLWPSSVPAYPSITVTPDEEQNLNHYIQVLENLVRSVPSGEPGREKKSNSPKHVYSIASK
GSKFKELVTHGDASTENDVLTNPISEETTTFPTGGFTPEIGKKKHTESTPFWSIKPNNVSIVLHAEEPYI
ENEEPEPEPEPAAKQTEAPRMLPVVTESSTSPYVTSYKSPVTTLDKSTGIGISTESEDVPQLSGETAIEK
PEEFGKHPESWNNDDILKKILDINSQVQQALLSDTSNPAYREDIEASKDHLKRSLALAAAAEHKLKTMYK
SQLLPVGRTSNKIDDIETVINMLCNSRSKLYEYLDIKCVPPEMREKAATVFNTLKNMCRSRRVTALLKVY

B) Correlation of CCT3 at peptide level with % D embryos.

MMGHRPVLVLSQNTKRESGRKVQSGNINAAKTIADIIRTCLGPKSMMKMLLDPMGGIVMTNDGNAILREI
QVQHPAAKSMIEISRTQDEEVGDGTTSVIILAGEMLSVAEHFLEQQMHPTVVISAYRKALDDMISTLKKI
SIPVDISDSDMMLNIINSSITTKAISRWSSLACNIALDAVKMVQFEENGRKEIDIKKYARVEKIPGGIIE
DSCVLRGVMINKDVTHPRMRRYIKNPRIVLLDSSLEYKKGESQTDIEITREEDFTRILQMEEEYIQQLCE
DIIQLKPDVVITEKGISDLAQHYLMRANITAIRRVRKTDNNRIARACGARIVSRPEELREDDVGTGAGLL
EIKKIGDEYFTFITDCKDPKACTILLRGASKEILSEVERNLQDAMQVCRNVLLDPQLVPGGGASEMAVAH
ALTEKSKAMTGVEQWPYRAVAQALEVIPRTLIQNCGAST IRLLTSLRAKHTQENCETWGVNGETGTLVDM
KELGIWEPLAVKLQTYKTAVETAVLLLRIDDIVSGHKKKGDDQSRQGGAPDAGQE

Potential phosphothreonine described in residue 459 (Underlined threonine)

C) Correlation of PRDX5 at peptide level with % D embryos.

MGLAGVCALRRSAGYILVGGAGGQSAAAAARRYSEGEWASGGVRSFSRAAAAMAPIKVGDAIPAVEVFEG
EPGNKVNLAELFKGKKGVLFGVPGAFTPGCSKTHLPGFVEQAEALKAKGVQVVACLSVNDAFVTGEWGRA
HKAEGKVRLLADPTGAFGKETDLLLDDSLVSIFGNRRLKRFSMVVQDGIVKALNVEPDGTGLTCSLAPNI
ISQL

Residues 57-145 (underlined) are missing in isoform 4 (P30044-4)

p< 0.05; p<0.1; p>0.1

Figure 3

BIOGRAPHY:

Dr. Meritxell Jodar is a postdoctoral researcher at Fundació Clínic per a la Recerca Biomedica and associate professor in the University of Barcelona. Her main research focus is the molecular study of sperm by different –omic technologies in order to develop molecular clinical biomarkers to improve the male infertility diagnosis and prognosis.