

# mRNA expression of oxidative-reductive proteins in boars with documented different fertility can identify relevant prognostic biomarkers

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## ARTICLE INFO

### Keywords:

ROS  
Oxidation  
Fertility  
AI  
Spermatozoa  
Pig

## ABSTRACT

Oxidative stress unbalance is a major factor causing impairment of sperm function and, ultimately, sperm death. In this study, we identified transcriptomic and proteomic markers for oxidative-related protectors from the generation of reactive oxygen species (ROS) in spermatozoa from breeding boars with documented high- or low-fertility. Particular attention was paid to glutathione peroxidases, and to transcripts related to DNA stabilization and compaction, as protamine and transition proteins. mRNA cargo analysis was performed using porcine-specific micro-arrays (GeneChip® miRNA 4.0 and GeneChip® Porcine Gene 1.0 ST) and qPCR validation. Differences between fertility-classed boars were ample among biomarkers; some upregulated only at protein level (catalase (CAT), superoxide dismutase 1 (SOD1) and glutathione proteins), or only at the mRNA level (ATOX1, Antioxidant Protein 1). In addition, protamines 2 and 3, essential for sperm DNA condensation and also transition proteins 1 and 2 (TNP1 and TNP2), required during histone-to-protamine replacement, were overexpressed in spermatozoa from high-fertile boars. This up-regulation seems concerted to reduce DNA accessibility to ROS attack, protecting the DNA. The upregulated intracellular phospholipid hydroperoxide glutathione peroxidase (GPx4), in high-fertile boars at mRNA level, can be considered a most relevant biomarker for fertility disclosure during sperm evaluation.

## 1. Introduction

Semen handling, including cooling or cryopreservation, leads to an increased production of Reactive Oxygen Species (ROS) that, above a threshold, impairs sperm homeostasis (Wang et al., 1997). Sperm ROS levels are leveled by the balance between ROS generation and ROS scavenging by antioxidants (Agarwal et al., 2014). If increased, oxidative damage can affect membrane integrity, DNA stability and mitochondrial function (John Aitken, 1995). Superoxide dismutase (SOD) (SOD1: soluble, SOD2: mitochondrial and SOD3: extracellular) catalyzes the dismutation of the superoxide radical into a molecular oxygen and hydrogen peroxide. If abundant, hydrogen peroxide leads to many types of cell damage (Hayyan et al., 2016). Catalase (CAT) is an important second ROS-scavenger, converting hydrogen peroxide into water and

oxygen. The ability to generate superoxide is linked to NADPH oxidase-like local activity (Alvarez et al., 1987; Vernet et al., 2001), and the addition of NADPH to human spermatozoa induces ROS in a dose-dependent manner (Said et al., 2005). The NADH-Ubiquinones, part of the respiratory chain complexes, are involved in the NADH transfer of electrons and the oxidation of NADH into its oxidized form (NAD<sup>+</sup>) (Galemou Yoga et al., 2021), and, ultimately, involved in the oxidation-reduction process, including post-translational protein modifications. Another suggested oxidative stress biomarker is glutathione (GSH), which oxidation is catalyzed by glutathione peroxidases. Together, these enzymatic systems are included when determining total antioxidant capacity (Alvarez-Rodríguez et al., 2013).

Boar sperm membranes, as those from other species, are rich in polyunsaturated fatty acids yielding a low resistance to oxidative stress

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<https://doi.org/10.1016/j.rvsc.2021.10.022>

Received 19 May 2021; Received in revised form 27 September 2021; Accepted 28 October 2021

Available online 3 November 2021

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(Brouwers et al., 2005). An increase in oxidative stress is suggested to involve nuclear DNA damage. Sperm DNA damage due to a deficiency in protamines has been described in sub-fertile men (Kazerooni et al., 2009). Moreover, TNP1 and TNP2 are, as the PRM1 and PRM2, involved in packing spermatid chromatin during human spermiogenesis (Jedrzczak et al., 2007). Sperm DNA fragmentation is being increasingly tested by swine artificial insemination (AI)-industry for its potential relation to fertility, and its correlation to DNA methylation (Khezri et al., 2019). In pigs, where the degree of nuclear compaction is very high, levels of PRM1 and PRM2 mRNA appeared significantly related to embryo cleavage rates (Hwang et al., 2013).

Conventional semen analysis is mostly restricted to assessment of sperm concentration and motility, but can include kinetics parameters (Peña et al., 2006), and assessment of the integrity of plasma and acrosome membranes, nuclear DNA damage, lipid peroxidation (Martínez-Pastor et al., 2010) and even apoptosis markers (Peña et al., 2003). Although this battery of methods attempts to mirror the potential fertility of the male after natural mating or AI (Rodríguez-Martínez, 2007), none appears to be best suitable biomarker. Markers based on gene expression measurement are considered, but since spermatozoa are described as transcriptionally and translationally silent except in their mitochondria (Gur and Breitbart, 2006), that diagnostic path has been largely neglected. While the presence of RNA has been described as remnants of spermatogenesis in boar (Yang et al., 2009), previous studies from our group found transcriptomic changes in pig spermatozoa in relation to fertility (Alvarez-Rodriguez et al., 2020).

Thus, the present study aimed to determine the relative abundance of mRNAs and proteins involved in ROS handling in ejaculated boar spermatozoa, and their relationship with fertility post-AI. Microarray-based technology, qPCR validation and western blotting were used to study whether mRNA expression and protein cargo differs in spermatozoa between high fertile and low fertile boars classed based on farrowing rate and litter size. The hypothesis tested was that the level of specific transcripts for enzymes and proteins relevant to combat/prevent damaging sperm oxidation is enriched in high-fertile breeding boars, becoming relevant prospective fertility biomarkers.

## 2. Materials and methods

The chemicals used in the experiments were of analytical grade. Unless otherwise stated, all reagents were acquired from Sigma-Aldrich (St. Louis, MO, USA).

### 2.1. Ethics statement

Animal husbandry and experimental handling were performed according to the European Directive 2010/63/EU, 22/09/2010 for animal experiments and current Swedish legislation (SJVFS 2017:40. Date: 06/2016) and approved by the Bioethics Committee of Murcia University (research code: 639/2012) and the “Regional Committee for Ethical Approval of Animal Experiments” (Linköpings Djurförsöksetiska nämnd) in Linköping, Sweden (permits no. 75–12, no. ID1400. Date: 12/2017) and Dnr 03416–2020 (03/2020)).

### 2.2. Ejaculated spermatozoa

Healthy, mature (1–2 years old) boars of Landrace and Large White breeds with proven fertility were housed in a commercial AI enterprise (Topigs Norsvin España, Calasparra, Murcia, Spain). All boars were fed with commercial feedstuff well-adjusted to nutritional requirements of adult AI boars, provided with water ad libitum and receiving the same management. Throughout all experiments, animals were handled carefully and in such a way as to avoid any unnecessary stress. The sperm-rich fraction (SRF) was collected using the gloved-hand method yielding a total of 28 ejaculates from seven different boars (one ejaculate/boar/month). All ejaculates used in the experiments fulfilled the

standards of sperm quantity and quality requirements for AI-doses ( $> 200 \times 10^6$  spermatozoa/mL, 70% motile spermatozoa, and 75% of morphologically normal sperm). The ejaculates used in the experiment were collected over a period of four months, while in the same period two other ejaculates were collected per week from the same boars and used to inseminate more than 1700 sows, whose fertility data in terms of farrowing rate (FR) and litter size (LS) were recorded. The raw fertility data were statistically corrected for parameters related to farm and sow (genetic line, weekday, age of semen, etc.) in order to isolate the contribution of the individual boars (direct boar effect) (Broekhuijsen et al., 2012). The direct boar effect on fertility was shown as the deviation of both the FR and LS of codified selected boars, including a correction from the boar population of the same genetic line. Thus, the seven boars were classified as having high ( $n = 4$ ) or low ( $n = 3$ ) fertility based in more than 100 artificial inseminations (up to 540) per boar, according to their FR and LS: high-fertility boars with a  $FR > 0.45$  and a  $LS > 0.15$ ; and low-fertility boars with  $FR < -0.12$  and  $LS < -0.18$ . In all cases, spermatozoa were separated from seminal plasma immediately after ejaculate collection by double centrifugation ( $1500 \times g$  at room temperature for 10 min, (Rotofix 32A, Hettich Zentrifugen, Tuttlingen, Germany)) and the resulting sperm pellets were stored at  $-80^\circ\text{C}$  (Ultra Low Freezer; Haier Inc., Qingdao, China). Four ejaculates from each boar were pooled and used for analyzing the differential gene expression profile, after RNA isolation.

### 2.3. RNA isolation

RNA was isolated using a commercially available kit designed to isolate cell-free RNAs for low quantity samples (RNeasy kit, Qiagen, Hilden, Germany), following the manufacturer protocol, with slight modifications (Alvarez-Rodriguez et al., 2020). Total RNA content and its quality was determined by NanoDrop® 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Only samples with high RNA quality (260/280 nm absorbance ratio of 1.8–2.2) were snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for its further analysis.

### 2.4. Microarray hybridization and scanning

Total RNA (75 ng) from each sample was used to make cDNA using GeneChip® Whole Transcript Plus reagent kit (Thermo Fisher Scientific, Göteborg, Sweden) following the manufacturer protocol (Alvarez-Rodriguez et al., 2020). 41  $\mu\text{L}$  of fragmented and labelled single stranded cDNA were mixed with 109  $\mu\text{L}$  of hybridization master mix to make a cocktail hybridization mix for a single reaction. The hybridization cocktail was then incubated first at  $99^\circ\text{C}$  for 5 min, followed by a descent to  $45^\circ\text{C}$  until loaded on the array chip (Affymetrix GeneChip® Porcine Gene 1.0 ST Array for gene expression; Thermo Fisher Scientific, Gothenburg, Sweden). A total of 130  $\mu\text{L}$  of the cocktail hybridization mix was loaded into each array chip and was incubated at  $45^\circ\text{C}$  under 60 rotations per min, for 16 h. The hybridized cartridge array chip was then unloaded and subjected to washing and staining using a GeneChip® Fluidics Station 450 (Thermo Fisher Scientific, Gothenburg, Sweden), to be finally scanned using the Affymetrix GeneChip® scanner GCS3000 (Thermo Fisher Scientific, Gothenburg, Sweden).

### 2.5. Bioinformatics and functional categorization

The intensity data of each array chip was processed using the robust multi-array average (RMA) normalization, computing average intensity values by background adjustment, quantile normalization among arrays and finally log2 transformation for extracting the expression values of each transcript in the probe set, and statistically analyzed implementing the official Transcriptome Analysis Console (TAC; version 4.0, Affymetrix, Thermo Fisher Scientific, Gothenburg, Sweden). A comparison was performed between the high ( $n = 4$ , and four ejaculates per each boar) and low ( $n = 3$ , and four ejaculates per each boar) fertility boars. The

differentially expressed genes (DEGs) that fulfilled the criteria (ANOVA  $p$ -value  $< 0.05$ ,  $-1 < \text{fold change} < 1$ ) were extracted as significantly up- or down-regulated transcripts. Molecular and biological functions as well as of overrepresentation analysis were identified using PANTHER GO (Mi et al., 2019).

## 2.6. qPCR validation

The synthesis of the first-strand cDNA was performed using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA), which consisted of 4 mg RNA in a final volume of 20 mL. After the synthesis, the samples were stored at  $-20^{\circ}\text{C}$  until further analyses.

A Quantitative Polymerase Chain Reaction (qPCR) was performed using the Real-Time PCR Detection System (CFX96; Bio-Rad Laboratories, Inc.; CA, USA). The reactions consisted of 0.5 mL of synthesized cDNA, 0.5 mL commercial gene-specific primers, 5 mL of PowerUp. SYBR. Green Master Mix (Applied Biosystems, CA, USA), and water to a final volume of 10 mL. The protocol was as follows: one cycle of uracil-DNA glycosylase (UDG) activation at  $50^{\circ}\text{C}$  for 2 min; one cycle of denaturation at  $95^{\circ}\text{C}$  for 2 min; and 40 cycles of denaturation at  $95^{\circ}\text{C}$  for 5 s, annealing/extension at  $60^{\circ}\text{C}$  for 30 s, and a melting curve at  $60$ – $95^{\circ}\text{C}$  ( $0.5^{\circ}\text{C}$  increments) for 5 s/step. Two technical replicates were used for each sample. The gene relative expression levels were quantified using the Pfaffl method (Pfaffl, 2001). Three housekeeping genes were initially used for cDNA normalization (*beta-ACTIN*, *GAPDH* and *vinculin*). After a preliminary analysis and validation of the results, only *GAPDH* was constantly expressed through the total RNA isolated from spermatozoa and was chosen for further analyses.

Commercial gene-specific PCR primers for boar spermatozoa samples were used (PrimePCR SYBR® Green Assay: *GAPDH*, *TNP1*, *TNP2*, *PRM1* and *PRM2*, *Sus scrofa*; Bio-Rad Laboratories, Inc.; CA, USA).

## 2.7. Protein expression by Western blotting

Total protein cargo from sperm samples from high fertility ( $n = 3$ ) and low fertility ( $n = 3$ ) boars were isolated following our previous protocol (Álvarez-Rodríguez et al., 2018). In brief, 100  $\mu\text{L}$  of RIPA buffer (Fisher Scientific, Gothenburg, Sweden), and protein inhibitor cocktail (Thermo Scientific Halt Protease Inhibitor Cocktail, 100 $\times$ , Fisher Scientific, Gothenburg, Sweden) were added to the eluted samples prior to keep the samples at  $4^{\circ}\text{C}$  for 1 h, vortexing each 10 min. After centrifugation of 13,000  $\times g$  for 10 min, proteins were quantified using an Invitrogen Qubit Protein Assay Kit (Fisher Scientific, Gothenburg, Sweden), following manufacturer's instructions. Protein suspensions (2.5  $\mu\text{g}$  protein/ $\mu\text{L}$ ) were denatured by heating at  $70^{\circ}\text{C}$  for 10 min and an aliquot (10  $\mu\text{L}$ ) of each protein suspension were loaded into 4%–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories AB, Solna, Sweden). After electrophoresis (150 V for 1 h) and transfer of the proteins to PVDF membranes (Bio-Rad Laboratories AB, Solna, Sweden) 100 V for 1 h, the membranes were blocked at room temperature for 1 h with TBS blocking solution (LI-COR Biosciences, Hamburg, Germany) and washed in phosphate buffered saline (TBS) (Bio-Rad Laboratories AB, Solna, Sweden) containing 0.1% Tween-20 (Sigma-Aldrich, Gothenburg, Sweden) (PBST). After three washes in PBST for 10 min, one membrane was incubated at  $4^{\circ}\text{C}$  overnight with the primary antibody (abcam, Amsterdam, Netherlands): GPx4 (ab41787, 1:1000), Glutathione (ab19534, 1:1000), CAT (ab50434, 1:1000), SOD (ab13498, 1:5000), ATOX1 (ab154179, 1:1000) and alpha tubulin (ab52866, 1:1000). The day after, the membrane was washed three times in TBST and incubated for 1 h with a dilution 1:15,000 of the secondary antibody (donkey anti-mouse IRDye 800 CW (925–32,210; donkey anti-rabbit IRDye 800 CW, LICOR Biosciences, Bad Homburg, Germany)) followed by extensive washing in PBST. The membranes were scanned using the Odyssey CLx (LICOR Biosciences, Bad Homburg, Germany), and images of the blots were obtained.

## 2.8. Statistical analysis of qPCR and Western blotting data

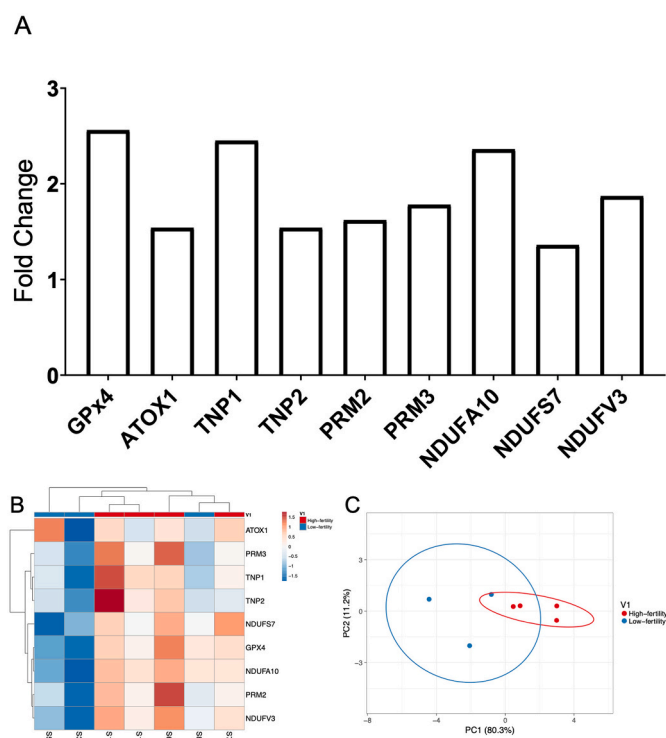
Normal distribution and homoscedasticity of the data were analyzed using the Shapiro–Wilk normality test and Levene's test. Non-normal data distribution was restored using Log(x) transformation prior to analysis. R-version 3.6.1. (R-Development Core Team, 2011) was used to conduct the statistical analyses, with nlme (Bates et al., 1998) to perform linear mixed effects (LME) models and multcomp (Hothorn et al., 2008) to perform pairwise comparisons adjusted by Tukey's test. The threshold for significance was set at  $p < 0.05$ . Data are presented as mean  $\pm$  SEM, unless otherwise stated. Our LME model included the fertility rate (low- and high-fertile boars) as fixed effects and the pool of samples as the random part of the model.

## 3. Results

### 3.1. Differentially expressed mRNA cargo between high and low fertile boars

Nine out of 73 genes selected through PANTHER analysis were differentially expressed (upregulated) ( $p < 0.05$ ) in high-fertile boars (Fig. 1.A and Supplementary Fig. 1).

Among the eight glutathione peroxidases included, only one (*GPx4*) was differentially expressed (upregulated in high-fertile boars). The antioxidant protein 1 (*ATOX1*), the transition proteins 1 and 2 (*TNP1*; *TNP2*), protamine 2 and 3 (*PRM2*; *PRM3*) were also upregulated ( $p < 0.05$ ) in high-fertile boars.



**Fig. 1.** Differential mRNA expression of oxidative-related genes in spermatozoa from high- vs. low-fertile boars analyzed by microarrays (A) ( $-1 > \text{FC} > 1$ ;  $p < 0.05$ ). Glutathione peroxidase 4 (*GPx4*), antioxidant protein 1 (*ATOX1*), transition protein 1 and 2 (*TNP1*; *TNP2*), protamine 2 and 3 (*PRM2*; *PRM3*), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42 kDa (*NDUFA10*), NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20 kDa (NADH-coenzyme Q reductase) (*NDUF57*) and NADH dehydrogenase (ubiquinone) flavoprotein 3, 10 kDa (*NDUFV3*). (B) Heat map of the different individual expression values from the differentially expressed mRNAs transcripts. (C) Principal component analysis (PCA) explaining the 91.5% of the total variation in the two first components.

None of the peroxiredoxins (PRDX1 to 5) were differentially expressed ( $p > 0.05$ ) (Supplementary Fig. 1.D).

Among the 40 NADH dehydrogenases (ubiquinone) (NDUF), only the NDUF 1 alpha subcomplex, 10, 42 kDa (NDUFA10), NDUF Fe—S protein 7, 20 kDa (NADH-coenzyme Q reductase) (NDUFS7) and NDUF flavo-protein 3, 10 kDa (NDUFV3) were upregulated ( $p < 0.05$ ) in high-fertile boars (Fig. 1.A. and Supplementary Fig. 1.E-H).

Hierarchical clustering (Fig. 1.B) for the differentially expressed mRNAs showed an acceptable classification of high and low fertility boars and, the Principal Component Analysis (PCA) explained the 91.5% of variation on the microarrays results (Fig. 1.C).

### 3.2. qPCR validation analysis

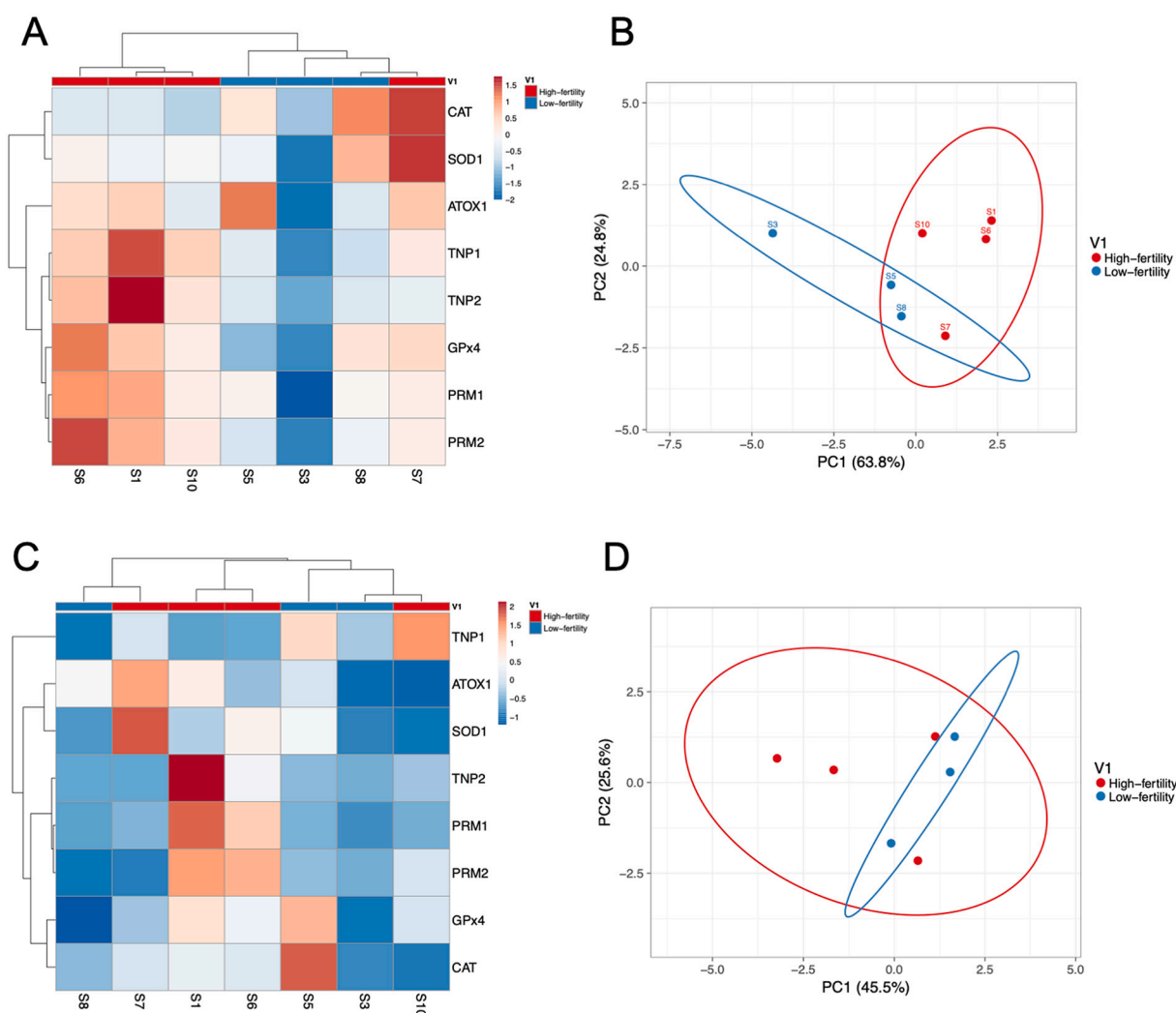
The heatmap showed a similar tendency of grouping for the microarrays results (Fig. 2.A) and the qPCR results (Fig. 2.C). The PCA explained the 88.6% of the variation on the microarrays results (Fig. 2.B) and the 71.1% in the qPCR results (Fig. 2.D). Moreover, almost all the transcripts analyzed for validation by qPCR, but PRM1 (microarrays results  $p = 0.065$ ) showed the same results (Supplementary Fig. 2).

### 3.3. CAT, SOD1 and glutathione protein cargo are overexpressed in high-fertility boars

The protein analysis of both the glutathione peroxidase 4 (GPx4) and the antioxidant protein 1 (ATOX1) resulted in non-significant differences ( $p > 0.05$ ) (Fig. 3). In contrast, catalase (CAT) and superoxide dismutase-1 (SOD1) were significantly increased in high-fertile boars (Fig. 4). Glutathione analysis at protein level also resulted in a significant ( $p < 0.05$ ) increase in high-fertile boars (Fig. 5).

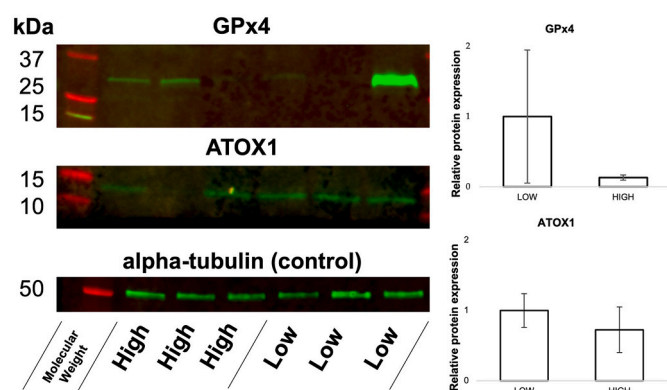
## 4. Discussion

The present study analyzed to what extent the relative load of mRNAs and proteins relevant to combat/prevent damaging sperm oxidation in ejaculated boar spermatozoa is related with their fertility post AI. Fertility among the breeding boars whose semen was classed in two subgroups according to their deviation in FR and LS from the average direct boar effect recorded by the boar population of the same genetic line: high- and low-fertility boars. To address our working hypothesis, we used different methodology microarray-based technology and PCR validation for mRNA expression analysis and western blotting



**Fig. 2.** Differential boar sperm mRNA expression analyzed by microarrays on catalase (CAT), superoxide dismutase 1 (SOD1), glutathione peroxidase 4 (GPx4), antioxidant protein 1 (ATOX1), transition protein 1 and 2 (TNP1; TNP2), protamine 2 and 3 (PRM2; PRM3), (A) Heat map of the different individual expression values from the differentially expressed mRNAs transcripts. (B) Principal component analysis (PCA) explaining the 88.6% of the total variation in the two first components, and qPCR (C) Heat map of the different individual expression values from the differentially expressed mRNAs transcripts. (D) Principal component analysis (PCA) explaining the 71.1% of the total variation in the two first components.





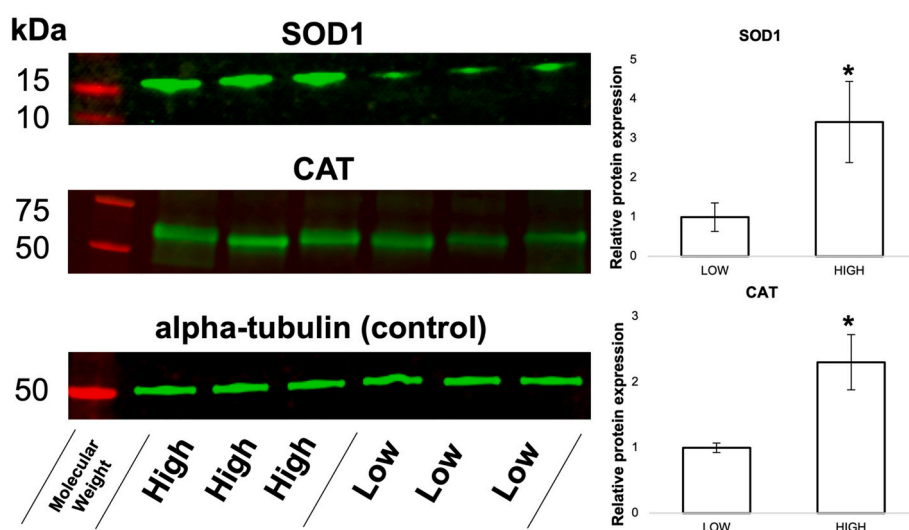
**Fig. 3.** Differential protein expression of boar sperm glutathione peroxidase 4 (GPx4) and the antioxidant protein 1 (ATOX1), using alpha-tubulin as endogenous control. Graphs to the right depict the relative protein expression for GPx4 and ATOX1 in high fertile boars (HIGH) relative to low fertile boars (LOW). Results are expressed in Mean  $\pm$  SEM.

for protein quantification.

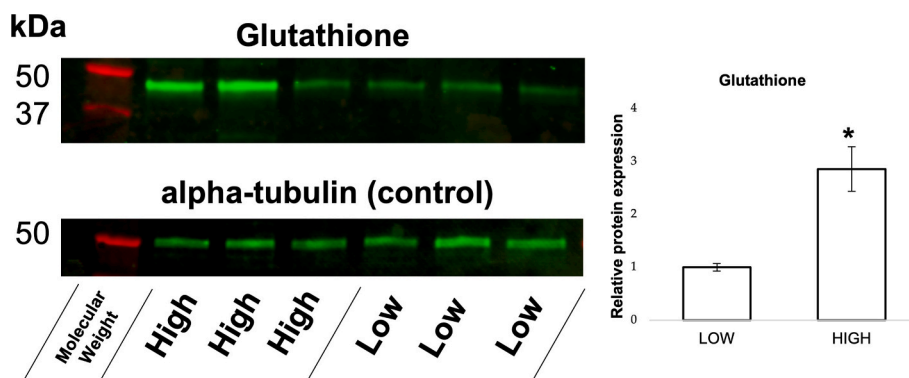
The overall results confirm the presence of a conserved upregulation of mRNA molecular markers as GPx4, ATOX1, PRM2 and PRM3 and TNP1 and TNP2, among others, in high fertility males. In addition, at

protein level, traditional markers of oxidative defense, as CAT, SOD1 and glutathione were upregulated in high fertility males.

The control of ROS levels in spermatozoa is complex and mediated by the balance between ROS-generation and ROS-scavenging by seminal plasma antioxidants mainly, but also by sperm-related systems (Agarwal et al., 2014). As example, the enzyme GPx4 is located on the post-acrosomal region and apical head region, as well as in sperm mid-piece and within the nuclear matrix (Drevet, 2006). GPx4 is an antioxidant enzyme, and as other GPx-family members, involved in the suppression of apoptotic cell death through reduction of ROS generation (Nomura et al., 1999) and which reduced presence leads to suboptimal fertility in humans (Imai et al., 2001). Our data agrees with these studies, implying an upregulation of GPx4 in high fertility males at mRNA level. However, in contrast to our results, GPx4 upregulation has been found in the spermatozoa of boars with small litter size after AI (Kwon et al., 2015a), whereas it is reduced during sperm capacitation (Kwon et al., 2014). Testicular GPx4 plays an important role in male fertility by affecting the sperm maturation and function (Ursini et al., 1999). Indeed, different isoforms play different roles, with the mitochondrial isoform being relevant for spermiogenesis, whereas the nuclear isoform is dispensable for fertility and the role of the cytosolic isoform is yet unclear (Flohé, 2007). These differences of action on sperm functionality could explain, at least in part, the high variability of GPx4 at protein level we detected, where one of the low-fertile boars



**Fig. 4.** Differential protein expression of boar sperm catalase (CAT) and superoxide dismutase 1 (SOD1), using alpha-tubulin as endogenous control. Graphs to the right depict the relative protein expression for CAT and SOD1 in high fertile boars (HIGH) relative to low fertile boars (LOW). Results are expressed in Mean  $\pm$  SEM. \* $p < 0.05$ .



**Fig. 5.** Differential protein expression of boar sperm glutathione, using alpha-tubulin as endogenous control. Graphs to the right depict the relative protein expression for glutathione in high fertile boars (HIGH) relative to low fertile boars (LOW). Results are expressed in Mean  $\pm$  SEM. \* $p < 0.05$ .

showed a clear overexpression of GPx4 protein. This finding demands further studies of the individual isoforms to determine the origin of the GPx4 protein.

The selenium-containing enzyme Glutathione Peroxidase (GPx) present in the male reproductive tract and spermatozoa (Drevet, 2006), has a relevant antioxidant effect preventing, by catalyzing the oxidation of GSH, the deleterious effects of a lipid peroxidation cascade (Storey et al., 1998). GPx expression failure in human spermatozoa was related to infertility (Foresta et al., 2002). Within the catalytic triad (SOD/GPx/CAT), the GPx-protein family is present in sperm maturation in the epididymis (Drevet, 2006), to probably ensure the best protection for spermatozoa. The current findings of GPx4 antioxidant upregulation in high fertile males at mRNA level, may confirm its key role.

Superoxide dismutase catalyzes the dismutation of the superoxide anion ( $O_2^{\cdot-}$ ) to produce hydrogen peroxide ( $H_2O_2$ ). To recycle hydrogen peroxide, two enzymatic activities are available: catalase, working mainly when high concentrations of  $H_2O_2$  are present, and glutathione peroxidase that use different substrates than  $H_2O_2$  and peroxiredoxins as  $H_2O_2$  scavengers and sensors in somatic cells (Rhee, 2006). Peroxiredoxins present a wide subcellular distribution (Banmeyer et al., 2004), when compared to catalase (restricted to peroxisomes) or GPxs (much less abundant) (Gong et al., 2012). Superoxide dismutase (SOD) activity is low and restricted to the little sperm cytoplasm (Aitken et al., 1996). Our data showed no differences ( $P > 0.05$ ) between high and low fertility boars, but a trend to downregulation at mRNA level, in all the five peroxiredoxins analyzed. There is an upregulation of SOD1 (both at mRNA and protein level), and CAT (at protein level), in high fertile boars. PRDX expression appears to be a suitable biomarker of litter size in pigs, with PRDX4 mRNA expression being highly predictive, with an accuracy of 95% (Ryu et al., 2020). In contrast, none of the peroxiredoxins (PRDX1 to 5) analyzed in the present study were differentially expressed. The difference might reside in the parameters of fertility explored between studies, since we based fertility both on farrowing rate and litter size.

Cytoplasmatic antioxidant protein 1 (ATOX1) protects from ROS (Stillman, 2007), keeps homeostasis (Hatori and Lutsenko, 2016) and has specific antioxidant functions, protecting cells against hydrogen peroxide-induced oxidative damage (Kelner et al., 2000). Homeostasis includes the entrance of copper (Cu) at the plasma membrane through the copper importer CTR1, to bind to thiol metabolites including glutathione. At least three different cytosolic copper chaperones (Atox1, Cox17, CCS) compete for Cu-GSH pool and sort Cu to specific destinations. Cu-Atox1 transfers copper to the copper transporting ATPases (ATP7A and ATP7B) in the membranes of trans-Golgi network (TGN) and secretory vesicles. Alternatively, SOD1 receives its copper cofactor predominantly from CCS, although in human cells glutathione can substitute for CCS (Carroll et al., 2004). The soluble copper chaperones (CCS, Atox1, Cox17, and possibly others) compete for copper in the cytosolic pool to facilitate copper supply to their specific target compartments. In a case of cytosol-mitochondria copper shuttles, copper transfer involves a redox reaction. Our results showed an increase in glutathione (protein level), ATOX1 (mRNA level), and SOD1 (protein level) in high fertility boars. It was recently demonstrated that copper chaperone function of Atox1 is required for vascular endothelial growth factor (VEGF)-induced angiogenesis via LOX activation (Chen et al., 2015). Previous studies by our group in pig (Álvarez-Rodríguez et al., 2020) and in rabbit (Gardela et al., 2020) showed an overexpression of VEGF-A in the female genital tract in response to the entrance of seminal plasma. However, the mechanism of action of ATOX1 mRNA in boar spermatozoa and an eventual link to effects in the female, requires further studies.

As for the second function, Atox1 was shown to protect yeast that was superoxide dismutase (SOD1)-deficient from oxidative damage induced by superoxide anions and hydrogen peroxide (Kelner et al., 2000). Moreover, Atox1 has, at least in mice, a double function: a copper chaperone and a positive regulator of SOD, with a suggested role in

oxidative stress modulation (Jeney et al., 2005). Once again, our results may confirm this relevant function as depicts by an increase in ATOX1 (mRNA level), and SOD1 (protein level) in high fertility boars.

NADH dehydrogenases, related to superoxide generation, were hereby overexpressed on the microarray's platform yet in only three out of 40 mRNA. NADH dehydrogenase contributes to site I of the mitochondrial electron transport chain (Ruiz-Pesini et al., 1998). In spermatozoa, NADH dehydrogenase is closely involved in tyrosine phosphorylation and sperm motility (Ruiz-Pesini et al., 1998). NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (NDUFS2) was significantly increased in the spermatozoa of boars producing small litter sizes (Kwon et al., 2015b,c). In contrast, our data showed an upregulation of NDUFA10, NSDUF57 and NDUFV3.

Complex I is the first enzyme of the mitochondrial respiratory chain which can be inhibited by interfering with the phosphorylation of NDUFS7 ultimately leading to mitochondrial dysfunction (Nuevo-Tapióles et al., 2020). Decreased levels of complex I (and of NDUFS7) increases mitochondrial ROS production, which may lead to increased assembly of the NLRP3-inflammasome complex at the mitochondria (Zhou et al., 2011). NDUFV3, is the first complex I subunit expressed in two isoforms with tissue-specific distribution (Dibley et al., 2017). Overall, the upregulation shown in the present study, might be related to a reduced activation of inflammasome-related genes.

Finally, boar spermatozoa present a high proportion of polyunsaturated fatty acids, increasing susceptibility for oxidative stress (Brouwers et al., 2005), and even related to damage to the chromatin/DNA (Li et al., 2018; Schneider et al., 2020). Our results found an overexpression of *PRM1* and *PRM2* by qPCR, which might relate to the preservation of DNA integrity by decreasing the chromatin access to deleterious agents, including ROS and damage-related to the chromatin/DNA (Brouwers et al., 2005; Li et al., 2018; Schneider et al., 2020). Moreover, the transition proteins 1 and 2 are also, as the *PRM1* and *PRM2*, involved in packing of spermatid chromatin during spermiogenesis (Jedrzejszak et al., 2007). Asthenozoospermic men present significantly lower levels of *TNP1* and *TNP2* transcripts in spermatozoa when compared to normozoospermic men. Our data agrees with this study, as both *TNP1* and *TNP2* mRNA transcripts are highly expressed in high fertility boars, compared with low fertility boars. Moreover, during sperm maturation, among several biochemical changes, some changes occur at chromatin level, with a progressive formation of intra- and intermolecular chromatin stabilizing disulphide bonds in the nuclear protamines at the epididymal middle segment (Rodríguez-Martínez et al., 1990) which strengthen DNA compaction (Balhorn, 2007), present in fertile boar spermatozoa (Rodríguez-Martínez, 2014). Thus, our results might reflect, through the overexpression of protamines and TNPs, a relation of boar fertility with the relative cargo of these transcripts in the mature spermatozoon.

## 5. Conclusion

The present study analyzed the sperm RNA- and protein-cargo of oxidative-related molecules involved in protecting spermatozoa of breeding boars with documented high- or low-fertility from the generation of reactive oxygen species (ROS). The most overexpressed mRNA found upregulated in high-fertile boars was the intracellular phospholipid hydroperoxide glutathione peroxidase (*GPx4*). *GPx4* ought to be considered a relevant fertility biomarker and its detection could be informative. However, before attempting its application during sperm evaluation and prior to using the semen in assisted reproductive techniques, higher numbers of boars ought to be explored attempting determination of intra- and inter-individual variability.

## Author contribution

Conceptualization, M.A.-R. and H.R.-M.; methodology, M.A.-R.; formal analysis, M.A.-R.; investigation, M.A.-R.; writing—original draft

preparation, M.A.-R.; writing—review and editing, M.A.-R., C.M.—S., J. R., H.R.-M.; project administration, M.A.-R and H.R.-M.; funding acquisition, H.R.-M., J.R., M.A.-R., C. M.-S. All authors have read and agreed to the published version of the manuscript.

## Funding

This research was funded by the Research Council FORMAS, Stockholm (Projects 2017–00946 and 2019–00288); grants AGL2015–69738-R, PID2019–108320RJ–100 and IJCI–2015–24380 funded by MCIN/AEI /10.13039/501100011033 (Spain) and FEDER funds (EU) and a a and the European Union's Horizon 2020 research and innovation program under the MSCA (grant agreement No 891663).

## Acknowledgements

Annette Molbaek and Åsa Schippert, for LiU core facility, for expert assistance when running the microarrays. To QualiSperm (AKYmed, Cheseaux-sur-Lausanne, Switzerland) for assisting with the computerized sperm motility and kinetics assessment. To Jaume Gardela and Mateo Ruiz, for their assistance with graphical content and critical discussion. To Linköping University for supporting the open access funding.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rvsc.2021.10.022>.

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