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ANALYSIS OF DNA POLYMORPHISMS AND EXPRESSION OF SELECTED GENES ASSOCIATED WITH DIFFERENT FREEZABILITY OF BOAR SPERMATOZOA

Doctoral thesis conducted

at the Department of Animal Biochemistry and Biotechnology

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ANALIZA POLIMORFIZMU DNA ORAZ EKSPRESJI WYBRANYCH GENÓW ZWIĄZANYCH ZE ZRÓŻNICOWANĄ PRZYDATNOŚCIĄ PLEMNIKÓW KNURA DO KRIOKONSERWACJI

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List of papers

This thesis is based on the studies of three papers, which are indicated in the text by their Roman numerals.

Paper I: Mańkowska A., Brym P., Paukszto Ł., Jastrzębski J.P., Fraser L. (2020). **Gene polymorphisms in boar spermatozoa and their associations with post-thaw semen quality**. *International Journal of Molecular Sciences* 21 (5), 1902. DOI: 10.3390/ijms21051902 (IF = 5.924; pts[2] = 140)

Paper II: Mańkowska A., Brym P., Sobiech P., Fraser L. (2022). **Promoter polymorphisms in *STK35* and *IFT27* genes and their associations with boar sperm freezability**. *Theriogenology* 189, 199–208. DOI: 10.1016/j.theriogenology.2022.06.023 (IF[1] = 2.923; pts[2] = 140)

Paper III: Mańkowska A., Gilun P., Zasiadczyk Ł., Sobiech P., Fraser L. (2022). **Expression of *TXNRD1, HSPA4L* and *ATP1B1* genes associated with the freezability of boar sperm**. *International Journal of Molecular Sciences* 23 (16), 9320. DOI: 10.3390/ijms23169320 (IF[1] = 6.208; pts[2] = 140)

[1]IF = Impact Factor indicates the year of the paper publication.

[2]pts = Points. The amount of points is based on the list of scientific journals published by the Ministry of Education and Science (MEiN) on 21st December, 2021.

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List of Abbreviations

AIs	-	artificial inseminations
APPL1	-	adaptor protein phosphotyrosine interaction PH domain and leucine zipper containing 1
ATP1B1	-	ATPase Na+/K+ transporting beta-1 polypeptide
BAM	-	binary alignment map
C/EBPα	-	CCAAT/enhancer binding protein alpha
CFAP52	-	cilia and flagella associated protein 52
CYP7B1	-	cytochrome P450 family 7 subfamily B member 1
dsSNPs	-	data base single nucleotide polymorphisms
ELK1	-	ETS transcription factor
EML5	-	Echinoderm microtubule associated protein like 5
FBXO16	-	F-box only protein 16
GATK	-	genome analysis tool kit
GAPDH	-	glyceraldehyde-3-phosphate dehydrogenase
GR-β	-	glucocorticoid receptor beta
HSPA4L	-	heat shock protein family A member 4 like
IFT27	-	intraflagellar transport 27
KASP	-	Kompetitive Allele Specific Polymorphism
KEGG	-	Kyoto Encyclopedia of Genes and Genomes
MAP3K20	-	mitogen-activated protein kinase 20
MS4A2	-	membrane spanning 4-domains A2
NCBI	-	National Center for Biotechnology Information
NFATC2	-	nuclear factor of activated T cells 2
NR3C1	-	nuclear receptor subfamily 3 group C member 1
OXSR1	-	oxidative stress responsive kinase
PCR	-	polymerase chain reaction
PLBD1	-	phospholipase B domain containing 1
PRICKLE1	-	prickle planar cell polarity protein 1
RAB3C	-	RAB3C, member RAS oncogene family
ROBO1	-	roundabout guidance receptor 1
SARS	-	seryl-tRNA synthetase
SCLT1	-	sodium channel and clathrin linker 1
SDS-PAGE	-	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SNP	-	single nucleotide polymorphism
SNVs	-	single nucleotide variants
SRA	-	Sequence Read Archive
STAR	-	spliced transcripts alignment to a reference
STK35	-	serine/threonine kinase 35
TFs	-	transcription factors
TRANSFAC	-	TRANScription FACtor
Tukey's HSD	-	Tukey’s Honest Significant Difference
TXNRD1	-	thioredoxin reductase 1
VEP	-	Variant Effect Predictor

Abstract

This thesis was performed in three studies. In the first study, variant calling of transcriptome sequencing data (RNA-Seq), which allowed the comparison of sperm transcriptome profiles from boars differing in freezability, was performed to identify single nucleotide polymorphisms (SNPs). Variant calling identified more than 1000 SNPs, in which most of them were detected at the 3′-untranslated regions (3′-UTRs). Forty SNPs were used for SNP genotyping by the KASP assay. Analysis showed associations of SNPs with some of the selected parameters of sperm functions following freezing-thawing. Analysis confirmed significant associations of *APPL1, PLBD1, FBXO16, EML5, RAB3C*, *OXSR1*, *PRICKLE1* and *MAP3K20* gene polymorphisms with the quality characteristics of frozen-thawed (FT) spermatozoa. It can be suggested that polymorphisms in the candidate genes are potential markers for post-thaw quality of boar semen.

In the second study, the 5′-flanking region sequences of *STK35,* a stress-related gene, and *IFT27,* a motility-related gene, were PCR amplified and analyzed by Sanger sequencing method to identify polymorphisms in spermatozoa of boars considered as having good and poor semen freezability (GSF and PSF, respectively). Bioinformatics was used to study the interactions of the identified SNPs with transcription factors (TFs) on the gene promoter activity. One SNP, rs327863835 (C > T) was detected in the *STK35* promoter, while two SNPs (rs337563873; A > T; rs331520020, T > C) were detected in the *IFT27* promoter. Significant allele frequency differences of *STK35* and *IFT27* promoter polymorphisms were observed between the freezability groups. It was predicted that *STK35* rs327863835 and *IFT27* rs331520020 resulted in the generation of additional binding sites for TFs, such as NFATC2, ELK1 and GR-β, which might regulate the transcriptional activity of the genes in either freezability group. Furthermore, protein expression of STK35 and IFT27 varied between the fresh and PT semen, however, IFT27 protein expression was more marked in FT

spermatozoa of the GSF group. It could be suggested that the allelic variants in *STK35* and *IFT27* can serve as potential markers for predicting the freezability of boar semen.

In the third study, it was investigated whether the expression of stress-related genes (*TXNRD1*, *HSPA4L* or *ATP1B1*) was associated with sperm cryotolerance. Higher relative mRNA expression levels of *TXNRD1* and *HSPA4L* were more marked in FT semen of the PSF group compared with the fresh semen. Likewise, HSPA4L protein expression was markedly higher in FT semen of the PSF group, while the fresh semen exhibited significantly higher expression of TXNRD1 protein in the GSF group. The significantly higher level of *ATP1B1* mRNA expression in the fresh semen of good freezability ejaculates could be used as a promising marker for semen freezability. Furthermore, variations in TXNRD1 and HSPA4L protein expression, and the electrophoretic protein profiles of FT semen between the freezability groups provide more insights into the role of sperm proteins in cryo-damage.

These studies indicate that the application of genetic markers for sperm freezability would contribute to the improvement in the cryopreservation of boar semen through the selection of individuals with high freezability ejaculates.

Key words: boar, spermatozoa, cryopreservation, polymorphisms, genes

Abstract (Polish)

Analiza polimorfizmu DNA oraz ekspresji wybranych genów związanych ze zróżnicowaną przydatnością plemników knura do kriokonserwacji

Przedłożona rozprawa doktorska została przygotowana w oparciu o wyniki uzyskane i zaprezentowane w cyklu trzech publikacji naukowych. W pierwszej z prac wykorzystano dane RNA-Seq uzyskane podczas porównania profili transkryptomicznych plemników knura o zróżnicowanej przydatności do kriokonserwacji nasienia. Wykorzystując analizę „variant calling", zidentyfikowano ponad 1000 polimorfizmów pojedynczych nukleotydów tzw. SNP i wykazano, że większość z nich znajdowała się w rejonach 3′-niepodlegających translacji (3′-UTR) sekwencjonowanych transkryptów plemnikowych. Do genotypowania populacji knurów wykorzystano technologię KASP. Czterdzieści z wykrytych SNP zostało wykorzystanych w badaniach populacyjno-asocjacyjnych knura rasy wielkiej białej polskiej, podczas których analizowano zależności pomiędzy genotypami poszczególnych loci a wybranymi parametrami charakteryzującymi jakość nasienia po rozmrożeniu. Analiza potwierdziła istotne związki pomiędzy polimorfizmem SNP w genach: *APPL1, PLBD1, FBXO16, EML5, RAB3C, OXSR1, PRICKLE1* i *MAP3K20* a cechami jakościowymi nasienia po zamrożeniu-rozmrożeniu. Zidentyfikowane SNP mogą być potencjalnymi markerami genetycznymi jakości i przydatności nasienia knura po kriokonserwacji.

W drugiej pracy analizowano związek pomiędzy polimorfizmami DNA w sekwencjach 5′-flankujących genów *STK35* i *IFT27* (funkcjonalnie związanych odpowiednio z odpowiedzią na bodźce stresowe oraz z ruchliwością plemników) a zróżnicowaną ekspresją białek STK35 i IFT27 w nasieniu knurów klasyfikowanych do grup o dobrej i złej przydatności do kriokonserwacji (grupy GSF i PSF). Za pomocą sekwencjonowania Sangera zidentyfikowano obecność tranzycji C > T (rs327863835) w promotorze genu *STK35* oraz dwóch substytucji A > T i T > C (rs337563873; rs331520020) w promotorze genu *IFT27*. Zaobserwowano statystycznie istotne różnice we frekwencjach poszczególnych alleli

analizowanych promotorów pomiędzy grupami knura GSF i PSF. Analizy bioinformatyczne wykazały, że mutacje *STK35* rs327863835 i *IFT27* rs331520020 mogą skutkować powstaniem dodatkowych miejsc wiązania dla czynników transkrypcyjnych takich jak: NFATC2, ELK1 i GR-β, co może różnicować poziom ekspresji *STK35* i *IFT27* w nasieniu knura o zróżnicowanej przydatności do technologii kriokonserwacji. Ponadto wykazano różnice w ekspresji białek STK35 i IFT27 pomiędzy nasieniem świeżym i mrożonym oraz że ekspresja białka IFT27 była najwyższa w plemnikach po rozmrożeniu z grupy GSF. Uzyskane wyniki sugerują, że warianty alleliczne w *STK35* i *IFT27* mogą być uznane za potencjalne markery genetyczne przydatności nasienia knura do kriokonserwacji.

W trzeciej pracy badano, czy ekspresja genów związanych ze stresem (*TXNRD1*, *HSPA4L* i *ATP1B1*) wpływa na kriotolerancję plemników knura. Na poziomie mRNA stwierdzono wyższą ekspresję genów *TXNRD1* i *HSPA4L* w nasieniu po rozmrożeniu z grupy PSF w porównaniu z nasieniem świeżym. Podobnie, ekspresja białka HSPA4L była wyraźnie wyższa w nasieniu po rozmrożeniu z grupy PSF, podczas gdy świeże nasienie wykazywało istotnie wyższą ekspresję białka TXNRD1 w grupie GSF. Istotnie wyższy poziom ekspresji mRNA *ATP1B1* w świeżym nasieniu o dobrej zamrażalności może być wykorzystany jako obiecujący marker kriotolerancji nasienia. Ponadto, różnice w ekspresji białek TXNRD1 i HSPA4L oraz profile elektroforetyczne białek nasienia po rozmrożeniu pomiędzy grupami GSF i PSF dostarczają nowych danych pozwalających ocenić ich znaczenie w procesach powstawania kriouszkodzeń plemników.

W podsumowaniu należy stwierdzić, że uzyskane wyniki wskazują na możliwość zastosowania markerów genetycznych w ocenie przydatności nasienia knura do kriokonserwacji i uzyskania postępu w tej dziedzinie na drodze selekcji osobników o korzystnych genotypach.

Słowa kluczowe: knur, plemniki, kriokonserwacja, polimorfizmy, geny

1. Introduction

1.1. Background

Cryopreservation of boar semen offers many benefits, such as the prolonged storage of high-value genetic materials (Holt et al. 2005; Yeste 2016). However, these benefits are often ignored due to poor cryo-survival of boar spermatozoa (Yeste 2016). Furthermore, despite efforts to improve the quality of frozen-thawed (FT) boar semen, inherent variability among individuals have contributed to poor sperm cryosurvival (Holt et al. 2005; Yeste 2016). Evidence has indicated that the inherent variations among boar affect the sperm's response to the freezing-thawing procedure (Holt et al. 2005; Fraser et al. 2014). Therefore, the pre-selection of individuals with high freezability ejaculates could improve the cryopreservation technology of boar semen.

1.2. Molecular markers for semen quality

Accumulating evidence has shown that genetic variations among boars have a significant effect on sperm freezability (Thurston et al. 2002; Holt et al. 2005). The utilization of genetic tools has provided more in-depth insight into the molecular mechanisms responsible for differences in the expression patterns and genetic variations in the pig reproductive tract (van Son et al. 2007; Dai et al. 2019; Fraser et al. 2020). Genetic analysis has demonstrated that polymorphisms in differentially expressed (DE) genes affect sperm quality traits in boars, and have been used as markers for semen quality (Wimmers et al. 2005). It has been reported that single nucleotide polymorphisms (SNPs) in the promoters of genes are associated with sperm functions (Cui et al. 2015; Brym et al. 2021). Moreover, DNA-binding sites for TFs can be affected by the presence of polymorphisms in the promoter region of a gene, thereby affecting its transcriptional activity (Buroker et al. 2016). In this study, the effects of promoter variants in *STK35* and *IFT27* (*RABL4*), which are implicated in various biological processes (Zhang et

al. 2017; Miyamoto et al. 2018), have been investigated in boar spermatozoa with different freezability. Moreover, *STK35,* a stress-related gene, is involved in spermatogenesis (Miyamoto et al. 2018), while *IFT27,* a motility-related gene, is associated with various sperm functions, such as flagellar assembly and metabolism (Zhang et al. 2017). It is noteworthy that the both *STK35* and *IFT27* were highly expressed in boar spermatozoa with poor freezability (Fraser et al. 2020).

Evidence has shown that cryo-induced changes in the gene expression profiles can be used as markers for semen quality (Valcarce et al. 2013; Chen et al. 2015; Yathish et al. 2017). It has been shown that cryopreservation reduced the expression profiles of Protamine 1 (*PRM1*) and Protamine 2 (*PRM2*) (Valcarce et al. 2013), whereas an overexpression of several DE genes was detected in FT bull spermatozoa (Chen et al. 2015; Yatish et al. 2017). In this thesis the effects of cryopreservation on the expression profiles of stress-related genes (*TXNRD1*, *HSPA4L* and *ATP1B1*) have been analyzed in boar spermatozoa. It is noteworthy that *TXNRD1*, *HSPA4L* and *ATP1B1* are involved in numerous sperm functions (Arnér and Holmgren 2000; Held et al. 2006; Thundathil et al. 2006; Liu et al. 2019). According to Moradi et al. (2018), the reduction of TXNRD1 levels in asthenozoospermic semen samples was concurrent with increased production of reactive oxygen species (ROS), apoptosis and the number of immature spermatozoa. Moreover, HSPA4L, as a member of the heat shock protein family (HSP110), is a mitochondrial stress protein that plays a role in spermatogenesis and osmotolerance (Held et al. 2006; Liu et al. 2019). The protein encoded by the *ATP1B1* gene belongs to the family of Na+/K+–ATPase pumps, and its high expression was associated with good freezability of the epididymal spermatozoa of the swamp buffalo (Salinas et al. 2022).

It is envisaged that molecular markers associated with genes controlling sperm freezability have the potential to improve the freezing protocol of boar semen.

2. Aims

It should be emphasized that a major factor that hinders the improvement in the cryopreservation boar semen is the selection of individuals whose semen could withstand the freezing-thawing procedure. Genetic differences in boars have been shown to contribute to the wide variations in cryotolerance among individuals. It seems, therefore, that the identification of potential molecular markers associated with sperm functions could improve the cryopreservation of boar semen.

Therefore, the specific aims were to:

i) employ variant calling analysis to identify SNPs in genes expressed in boar spermatozoa (**Paper I**),

ii) validate selected SNP markers using the KASP genotyping assay (**Paper I**),

iii) investigate the associations of gene polymorphisms with the quality characteristics of FT spermatozoa (**Paper I**),

iv) identify polymorphisms in the promoters of *STK35* and *IFT27* genes of spermatozoa with good and poor freezability, and to predict their effect on the binding ability of TFs (**Paper II**),

v) analyze *TXNRD1*, *HSPA4L* and *ATP1B1* mRNA expression in the fresh pre-freeze (PF) and FT spermatozoa from boars differing in freezability (**Paper III**), and

vi) determine the protein expression of the analyzed genes in the fresh PF and FT spermatozoa (**Paper II** and **Paper III**), and to identify the electrophoretic profiles of the proteins in ejaculates with different freezability (**Paper III**).

3. Materials and Methods

3.1. Animals and semen collections

Ejaculates were collected from forty Polish large white (PLW) boars. Most of the ejaculates were collected from boars stationed at the Cryopreservation laboratory, Faculty of Animal Bioengineering, Department of Animal Biochemistry and Biotechnology, UWM in Olsztyn (**Paper I**). Ejaculates were collected from eleven and ten boars for the studies in **Paper II** and **Paper III**, respectively. **Paper II** is a follow-up study in which 33 ejaculates were collected from 5 boars considered having good semen freezability (GSF), while 36 ejaculates were collected from 6 boars classified as having poor semen freezability (PSF) (Brym et al. 2021).

3.2. Cryopreservation procedure

Semen was frozen according to a cryopreservation protocol (Fraser and Strzeżek, 2007; Fraser et al. 2010; Wasilewska and Fraser 2017). All samples (500×10^6 spermatozoa/mL) were frozen in a programmable computer freezer (Ice Cube 1810, SY-LAB), prior to storage in liquid nitrogen. Frozen samples were thawed in a water bath for 60 sec at 50 °C for post-thaw sperm analysis. Prior to sperm quality assessment, samples were held in a water bath for 10 min at 37 °C.

3.3. Laboratory assays for semen evaluation

3.3.1. Sperm motility analysis

Total sperm motility (TMOT) was assessed microscopically (subjectively). Diluted semen samples were placed on pre-warmed slide and assessed under a light microscope at 200× magnification (Olympus BX 40) equipped with an attached heated stage. Besides TMOT and progressive motility (PMOT), the computer-assisted semen analysis (CASA) system

(HTR-IVOS 12.3, Hamilton Thorne Biosciences) was used analysed various sperm motion characteristics (Fraser et al. 2020).

3.3.2. Sperm membrane integrity assessment and lipid peroxidation

Sperm mitochondrial membrane potential (MMP) was assessed with the fluorescent lipophilic cation JC-1 and propidium iodide (PI) fluorescent dyes (Dziekońska et al. 2009; Fraser et al. 2014), while the sperm plasma membrane integrity (PMI) was assessed with the SYBR-14 and PI fluorescent probes, using the Live/Dead Sperm Viability Kit (Molecular Probes) (Garner and Johnson 1995). The percentage of spermatozoa with normal apical ridge (NAR) acrosome integrity was assessed, according to a previously described method (Fraser et al. 2007; Wasilewska and Fraser 2017). The Comet assay was used to monitor sperm DNA fragmentation (Fraser and Strzeżek 2007; Fraser et al. 2010). Sperm lipid peroxidation was determined spectrophotometrically by malondialdehyde (MDA) production (Strzeżek et al. 2000; Fraser et al. 2014).

3.4. DNA and RNA isolation from spermatozoa

Genomic DNA was isolated from the fresh PF spermatozoa of forty boars, using the protocol of the Sherlock AX Purification kit (A&A Biotechnology, Gdynia, Poland). The DNA samples were treated with a precipitation enhancer isopropanol, before being washed twice in 70% ethanol and air-dried at room temperature. The DNA yields and quality were analysed with the Nanodrop Spectrophotometer (ND-1000, NanoDrop, Thermo Fisher Scientific Inc.) and the samples stored at −20 °C, until further analysis.

Total RNA was isolated from the fresh PF and FT spermatozoa from ten boars (5 boars each of the GSF and PSF groups, respectively), according to a previously published study (Fraser et al. 2020). Sperm cells (150×10^6 spermatozoa/mL) were lysed in a Lysis Buffer

(PureLink RNA mini kit) and the RNA pellets were treated with TRIzol (Invitrogen, Thermo Fisher Scientific Inc.). The PureLink RNA mini kit (Invitrogen, Thermo Fisher Scientific Inc.) was used to extract RNA, according to the manufacturer's instructions. The quality of total RNA was examined by a Nanodrop Spectrophotometer (ND-1000, NanoDrop, Thermo Fisher Scientific Inc.).

3.5. SNP identification and validation

3.5.1. Variant calling and filtering procedure

RNA-Seq datasets that were submitted to the NCBI-SRA database (Bioproject: PRJNA415904; accession number SRP121647) were used for variant calling analysis (**Paper I**). The RNA-Seq data represented six PLW boars (three boars each of the GSF and PSF groups, respectively), as described in a previous study (Fraser et al. 2020). The clean reads of RNA-Seq were mapped to the *Sus scrofa* reference genome from Ensembl (genome-build Scrofa 11.1.91), using the STAR software (Dobin et al. 2013). The BAM alignment files were processed, and SNV calling analysis was performed by the Picard and GATK tool (McKenna et al. 2010). Further processing, filtering and SNV validation were performed with the rMATS-DVR software (Wang et al. 2017). The detected variants were annotated using the SnpEff software v.4.1 and VEP Ensembl to classify the SNVs (Cingolani et al. 2012; McLaren et al. 2016). For validation purposes, 40 SNPs were genotyped, using the KASP genotyping assay (LGC Genomics Ltd., Trident Industrial Estate, Hoddesdon, Hertfordshire, UK).

3.5.2. SNP validation

Genomic DNA samples, isolated from boar spermatozoa, were used to genotype 40 SNPs, using the KASP genotyping assay (LGC Genomics Ltd., Trident Industrial Estate,

Hoddesdon, Hertfordshire, UK). The data were analyzed with the Kluster-caller software and SNP Viewer (http://www.lgcgroup.com).

3.6. Analysis of the 5′-flanking sequences of *STK35* and *IFT27* genes

For **Paper II**, preparation and analysis of the5′-flanking sequences of *STK35* and *IFT27* were performed, according to a previously published study (Brym et al. 2021). Amplification products, obtained by PCR analysis, were analyzed by bidirectional Sanger sequencing (Genomed S.A. Company, Warsaw, Poland). DNA polymorphisms were identified by the Clustal W multiple alignment program (Larkin et al. 2007), using the BioEdit Sequence Alignment Editor ver. 7.2.6 (Hall 1999).

Analysis of SNP sequences in *STK35* and *IFT27* promoters was performed, using the ALGGEN via PROMO analysis tool (Messeguer et al. 2002; Farré et al. 2003), which utilized the TRANSFAC database (ver. 8.3) to construct the specific binding site weight matrices for the predictions of the transcription factor binding sites (Matys et al. 2006). The threshold for the maximum matrix dissimilarity rate was 5% (Quandt et al. 1995).

3.7. RT-qPCR analysis

The RT-qPCR (reverse transcription-quantitative PCR) analysis (**Paper III**) was used to quantify relative mRNA expression of *TXNRD1, HSPA4L* and *ATP1B1*, according to a previously published study (Koziorowska-Gilun et al. 2015). Amplifications were performed in a Real-Time PCR system (ABI 7900 HT, Applied Biosystems), using the master mix volume that comprised the SYBR Green mix, forward and reverse primers and template cDNA. The relative mRNA quantifications were performed by comparing the genes of interest with the reference gene, GAPDH (Zeng et al. 2014), and are expressed as arbitrary units, using the Real Time PCR Miner algorithm (Zhao and Fernald 2005).

3.8. KEGG pathways and GO enrichment analysis

Enrichment analysis was performed with the Kobas annotation tool (v3.0), g:Profiler online tool or Blast2Go Pro software (v.5.2.5) to identify functional KEGG pathways and Gene Ontology (GO) categories of biological process (BP), molecular function (MF) and cellular components (CC) of the candidate genes with polymorphisms (**Paper I**), or *TXNRD1, HSPA4L* and *ATP1B1* genes (**Paper III**).

3.9. Western blotting analysis

Western blotting analysis was performed in sperm extracts (50×10^6 spermatozoa/mL) to quantify protein expression of STK35, IFT27, TXNRD1, HSPA4L and ATP1B1 (Fraser et al. 2020). The MultiAnalyst 1.1 software (Bio-Rad Laboratories) was used to analyze protein bands and normalization of the protein expression (Vilagran et al. 2013).

3.10. Statistical analysis

Statistical analysis was performed with the IBM SPSS Statistics software package (IBM SPSS Statistics for Windows, IBM Corp.) and StatSoft Statistica software package (StatSoft Incorporation). The general linear modeling (GLM) procedure was used for ANOVA analysis of the sperm parameters, using three Models (**Paper I** and **Paper II**). For **Paper I**, the program GENEPOP v.4.7.2 software package (Rousset 2008) was used to calculate the allele and genotype frequencies (Fisher's exact test), Hardy-Weinberg equilibrium (HWE) for the loci (Fisher's exact test and chi-square test - $\chi 2$), and to determine the population differences between the GSF and PSF groups. Data were analysed with ANOVA (followed by Tukey's HSD test) or with the Kruskal-Wallis ANOVA with a multiple comparison test (**Paper II** and **Paper III**). For **Paper II** differences in the allele frequencies between the GSF and PSF groups were compared, using the Pearson's chi-square test (χ^2) (Brym et al. 2021).

Boars were allocated to the GSF and PSF groups, according to post-thaw TMOT assessment (>30% and <30% TMOT, respectively). Descriptive variables are presented as the mean ± SEM, and significant differences between the GSF and PSF groups were compared using the Student-t test or the Mann-Whitney U test ($P < 0.05$).

4. Results and Summary of Papers

4.1. Paper I

Mańkowska A., Brym P., Paukszto Ł., Jastrzębski J.P., Fraser L. (2020). **Gene polymorphisms in boar spermatozoa and their associations with post-thaw semen quality**

In this study variant calling analysis of RNA-Seq datasets was used to identify SNPs in genes expressed in boar spermatozoa and to investigate their associations with post-thaw semen quality. Following multi-filtering analysis of the dbSNPs, 1371 SNP variants were annotated, in which most of the putative polymorphisms were predicted to be located at the 3′-untranslated regions (3′-UTRs) followed by the 5′-untranslated regions (5′-UTRs). In addition, approximately 5.4% missense polymorphism was detected in candidate genes by variant calling. Analysis of SNP abundance in the different functional gene categories showed that the GO terms were related to several biological processes, such as response to stress (*OXSR1*), motility (*EML5*), and metabolism (*PLBD1*).

In the experimental design of this study candidate genes that were related to semen traits, based on their role in sperm functions and the polymorphism coverage, were used as criteria to select SNPs for KASP genotyping. Thirty-seven SNPs were validated by the KASP genotyping assay. There were variations in the frequency distributions of the alleles of the candidate genes. Irrespective of the freezability group, higher frequencies of the alleles G and A were observed

in *CYP7B1* (0.90) and *CFAP52* (0.72) gene loci, respectively. Likewise, there were higher frequencies of the alleles C and T in *EML5* (0.72) and *ROBO1* (0.87) gene loci, respectively.

ANOVA analysis showed that polymorphisms (*CYP7B1* rs338842672, *RAB3C* s81210636, *OXSR1* rs339379734, *FBXO16* rs341614458, *PLBD1* rs321497623, *EML5* rs345056502, *SARS* rs344846507 and *PRICKLE1* rs694366781) in the candidate genes were significantly ($P < 0.05$) associated with post-thaw semen quality. Twenty one boars were allocated to the GSF group, while 19 boars were assigned to the PSF group, according to post-thaw TMOT. Besides SNP genotype (polymorphisms), ANOVA showed that differences in freezability (GSF and PSF groups) significantly ($P < 0.05$) affected the quality of FT semen. It was found that *SCLT1* rs337913978, *MAP3K20* rs340643892, *MS4A2* rs339836492 and *ROBO1* rs331568674 were significantly ($P < 0.05$) associated with motility, PMI, acrosome integrity, LPO and DNA fragmentation of FT spermatozoa of either freezability group.

In this study, the effect of most of putative polymorphisms that were at the 3′-UTR regions on sperm cryosurvival is not fully understood. Among the variants at the 3′-UTR region, *OXSR1* rs339379734 and *SCLT1* rs337913978 showed significant associations with post-thaw semen quality. Also, eight synonymous SNPs that were detected in *CYP7B1*, *FBXO16*, *RAB3C*, *SARS*, *MAP3K20, PLBD1, EML5* and *PRICKLE1* genes showed significant ($P < 0.05$) associations with post-thaw semen quality. These SNPs could be linked to causative mutations, and might have functional effects on the male reproductive traits. Likewise, two missense SNPs in *APPL1* and *MS4A2* genes were significantly ($P < 0.05$) associated with the quality characteristics of FT spermatozoa. It is apparent that the missense polymorphism in either *APPL1* (a novel predicted SNP) or *MS4A2* rs339836492 could have a marked effect on the gene protein expression, and might affect the sperm's response to the cryopreservation conditions. The results of this study have confirmed that polymorphisms in the candidate genes are

associated with sperm cryo-survival and could be used as markers to improve the post-thaw quality of boar semen.

4.2. Paper II

Mańkowska A., Brym P., Sobiech P., Fraser L. (2022). **Promoter polymorphisms in *STK35* and *IFT27* genes and their associations with boar sperm freezability**

In this study it was hypothesized that SNPs in the 5′-flanking regulatory regions of *STK35* and *IFT27* genes could contribute to differences in semen freezability. It was also predicted the SNP interactions with TFs could affect the gene promoter activity. A single SNP, rs327863835 (C > T) in *STK35* promoter and two SNPs (rs337563873, A > T; rs331520020, T > C) in *IFT27* promoter were detected by Sanger sequencing. Marked ($P < 0.05$) differences in the genotypes of allelic variants in either *STK35* or *IFT27* promoter region were found between the freezability groups. *STK35* rs327863835 (C > T) showed a higher ($P < 0.05$) frequency of the C allele in the GSF group, while a higher ($P < 0.05$) frequency of the T allele was observed in the PSF group. *IFT27* rs337563873 (A > T) exhibited higher ($P < 0.05$) frequencies of the A and T alleles of the PSF and GSF groups, respectively. Three genotypes (C/C, T/C and T/T), with different frequencies of the T and C alleles in the freezability groups, were detected in *IFT27* rs331520020.

In silico analysis showed that SNPs in the *STK35* and *IFT27* promoter regions could resulted in the addition (+) of a new transcription factor (TF) binding site or the removal (–) of the original TF. Analysis showed that *STK35* rs327863835 resulted in the generation of a new TF binding site for NFATC2, while *IFT27* rs331520020 resulted in the addition of two new binding sites, ELK1 and NR3C1 (GR-β), and the elimination of the original binding site for C/EBPα. Furthermore, western blotting analysis showed lower ($P < 0.05$) expression

of STK35 protein in FT spermatozoa compared with the fresh PF spermatozoa, regardless of the freezability group. However, significantly higher ($P < 0.05$) post-thaw expression of IFT27 protein in the good freezability ejaculates was not reflected in improved quality characteristics of FT spermatozoa.

The results of this study show that polymorphism in *STK35* promoter was more marked in PSF group, while *IFT27* promoter polymorphisms were more noticeable in the GSF group. Polymorphisms affected the DNA-binding sites for TFs in *STK35* and *IFT27* promoters, and could affect the transcriptional activity of the genes, which might predispose spermatozoa to varying susceptibility to cryo-damage. The results indicate that the allelic variants in *STK35* and *IFT27* could be considered as potential markers for predicting the freezability of boar semen.

4.3 Paper III

Mańkowska A., Gilun P., Zasiadczyk Ł., Sobiech P., Fraser L. (2022). **Expression of *TXNRD1, HSPA4L* and *ATP1B1* genes associated with the freezability of boar sperm**

In this study the effect of the freezing-thawing procedure on mRNA and protein expression levels of *TXNRD1, HSPA4L* and *ATP1B1* genes in boar spermatozoa from the GSF and PSF groups. The mRNA expression levels of *TXNRD1, HSPA4L* and *ATP1B1* varied markedly in the fresh PF or FT spermatozoa among the boars. This phenomenon was more marked in the poor freezability ejaculates. It was found that FT spermatozoa exhibited significantly higher ($P < 0.05$) *TXNRD1* mRNA expression compared with the fresh PF spermatozoa of the PSF group, while *HSPA4L* mRNA expression levels in the FT spermatozoa of either freezability group were significantly higher ($P < 0.05$) in the fresh PF spermatozoa of the PSF group. Furthermore, *ATP1B1* mRNA expression levels in the fresh PF spermatozoa of

GSF group were higher ($P < 0.05$) than in the PSF group. It was found that *TXNRD1, HSPA4L* and *ATP1B1* were associated with several biological processes and KEGG pathways, such as cellular homeostasis, novo centriole assembly, selenocompound metabolism and FoxO signaling pathway.

Variations in the expression levels of TXNRD1 (70 kDa), HSPA4L (~94 kDa) or ATP1B1 (~63 kDa and ~70 kDa) were observed in the fresh PF and FT spermatozoa. Fresh PF and FT spermatozoa of the PSF group showed higher ($P < 0.05$ and $P < 0.01$, respectively) expression of TXNRD1 protein than in the fresh PF spermatozoa of the GSF group. Irrespective of the freezability group, FT spermatozoa exhibited significantly higher HSPA4L protein expression than the fresh PF spermatozoa from the poor freezability ejaculates. Cryopreservation caused a gradual decline in the relative expression of ATP1B1 protein, being markedly reduced ($P < 0.05$) in FT spermatozoa of the PSF group compared with the fresh PF spermatozoa. In addition, SDS-PAGE and densitometric analyses showed variations in the protein abundance between the fresh PF and FT spermatozoa of the freezability groups, being markedly higher in FT spermatozoa of the GSF group. Several additional proteins with different molecular weights were observed in the profiles of the FT spermatozoa from most of the boars of the GSF group. It can be suggested that the variations in the post-thaw expression levels of the TXNRD1 or HSPA4L protein provide more insights into the molecular mechanisms involved in sperm cryo-damage.

5. Conclusions

The following conclusions from this thesis are as follows:

I. KASP genotyping assay and SNP association analysis with sperm traits confirmed that allelic variants in *APPL1, PLBD1, FBXO16, EML5, RAB3C*, *OXSR1, PRICKLE1* and *MAP3K20* genes are promising markers for post-thaw semen quality.

II. Polymorphisms in the promoter regions of the differentially expressed *STK35* and *IFT27* in good and poor freezability ejaculates resulted in the generation of additional DNA-binding sites for transcription factors, which might regulate the transcriptional activity of the genes. The allelic variants in *STK35* and *IFT27* could be considered as potential markers for predicting the freezability of boar spermatozoa.

III. Relative mRNA *ATP1B1* expression in the fresh semen analyzed by RT-qPCR could be used to select high freezability ejaculates.

IV. Significant variations in the relative protein expression of STK35, IFT27, TXNRD1, HSPA4L and ATP1B1 between the good and poor freezability ejaculates reaffirm the potential roles of these proteins in sperm cryotolerance.

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Papers I-III

Paper I

Mańkowska A., Brym P., Paukszto Ł., Jastrzębski J.P., Fraser L. (2020). **Gene polymorphisms in boar spermatozoa and their associations with post-thaw semen quality**. *International Journal of Molecular Sciences* 21 (5), 1902.

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Article

Gene Polymorphisms in Boar Spermatozoa and Their Associations with Post-Thaw Semen Quality

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Abstract: Genetic markers have been used to assess the freezability of semen. With the advancement in molecular genetic techniques, it is possible to assess the relationships between sperm functions and gene polymorphisms. In this study, variant calling analysis of RNA-Seq datasets was used to identify single nucleotide polymorphisms (SNPs) in boar spermatozoa and to explore the associations between SNPs and post-thaw semen quality. Assessment of post-thaw sperm quality characteristics showed that 21 boars were considered as having good semen freezability (GSF), while 19 boars were classified as having poor semen freezability (PSF). Variant calling demonstrated that most of the polymorphisms (67%) detected in boar spermatozoa were at the 3′-untranslated regions (3′-UTRs). Analysis of SNP abundance in various functional gene categories showed that gene ontology (GO) terms were related to response to stress, motility, metabolism, reproduction, and embryo development. Genomic DNA was isolated from sperm samples of 40 boars. Forty SNPs were selected and genotyped, and several SNPs were significantly associated with motility and membrane integrity of frozen-thawed (FT) spermatozoa. Polymorphism in *SCLT1* gene was associated with significantly higher motility and plasma membrane integrity of FT spermatozoa from boars of the GSF group compared with those of the PSF group. Likewise, polymorphisms in *MAP3K20*, *MS4A2*, and *ROBO1* genes were significantly associated with reduced cryo-induced lipid peroxidation and DNA damage of FT spermatozoa from boars of the GSF group. Candidate genes with significant SNP associations, including *APPL1*, *PLBD1*, *FBXO16*, *EML5*, *RAB3C*, *OXSR1*, *PRICKLE1*, and *MAP3K20* genes, represent potential markers for post-thaw semen quality, and they might be relevant for future improvement in the selection procedure of boars for cryopreservation. The findings of this study provide evidence indicating that polymorphisms in genes expressed in spermatozoa could be considered as factors associated with post-thaw semen quality.

Keywords: freezability; RNA-Seq; variant calling; genotype; SNPs

1. Introduction

Cryopreservation of semen allows for prolonged storage of genetically important reproductive traits through the use of assisted reproductive techniques (ARTs), such as artificial insemination (AI) [1,2]. Due to poor post-thaw semen quality and reduced fertility, the widespread application of frozen-thawed (FT) boar semen in the AI practices is limited compared to liquid-stored semen [2].

Selection of high proportions of viable FT spermatozoa is the major challenge in the pig AI industry [3]. Moreover, the selection of a high number of functionally viable FT boar spermatozoa is required to minimize economic loss and increase the worldwide application of FT semen in AI technologies [1,2].

Analysis of different sperm attributes has confirmed that cryo-induced damage to spermatozoa differs among individual boars, suggesting varying responses to the freezing–thawing process [4,5]. Evidence has indicated that the freezability of boar semen is influenced by genetic factors [4,6]. Recent developments in high-throughput sequencing techniques, such as transcriptome sequencing (RNA-Seq), have enabled a thorough analysis of gene expression and genetic variations in the pig reproductive tract [7]. Our recent study has confirmed that there are differences in the transcriptome profiles of boar spermatozoa [8]. Furthermore, it has been confirmed that boars with poor freezability ejaculates are characterized by an overexpression of differentially expressed genes (DEGs) that are mainly associated with inflammation and apoptosis, which increase the sperm susceptibility to cryo-induced damage [8]. Previous studies confirmed that genes enriched in the cytokine–cytokine receptor interaction and inflammatory response-related pathways predisposed boar sperm to cryo-induced damage [9,10]. Hence, transcriptome alterations in spermatozoa have been considered to be one of the main factors affecting the cryo-survival of boar semen [8–10]. It should be emphasized that variant calling procedure based on RNA-Seq data has been suggested to be an important screening tool to identify polymorphisms in differentially expressed genes [7] and potential genetic markers associated with production traits in the pig industry [11].

Several authors have reported an increasing number of markers related to reproduction traits in pigs [12–14]. Advances in molecular genetics have resulted in the identification of several single nucleotide polymorphisms (SNPs) that are associated with the quality parameters of boar spermatozoa (including motility and morphology), and there has been increasing interest to understand the molecular processes that affect the sperm phenotype traits [12,15]. Evidence has shown that polymorphisms in boar sperm could be used as markers for semen quality [12,15,16]. However, to date, there is limited information about the associations of SNPs with post-thaw quality of boar semen. To our knowledge, no study has yet explored the associations between SNP markers and post-thaw quality semen of the Polish large white (PLW) boars. The objectives of this study were to i) identify polymorphisms in candidate genes (CGs) of boar spermatozoa using variant calling analysis of RNA-Seq dataset, ii) identify the functional categories of SNP abundance in the CGs by gene ontology (GO) enrichment analysis, iii) validate the selected SNPs using the Kompetitive Allele Specific Polymorphism (KASP) genotyping assay, and iv) explore the associations of gene polymorphisms with post-thaw (PT) semen quality.

2. Results

2.1. Assessment of Semen Quality

Repeated measures analysis of variance (ANOVA) did not show significant differences ($p > 0.05$) among the boars, with respect to fresh, pre-freeze semen quality. However, ANOVA results demonstrated that boar was a significant ($p < 0.001$) factor affecting post-thaw motility, mitochondrial membrane potential (MMP), plasma membrane integrity (PMI), normal apical ridge (NAR) acrosome integrity, DNA fragmentation, and lipid peroxidation (LPO). The quality of fresh, pre-freeze, and post-thaw semen for 40 boars is shown in Table 1. Boars showing more than 30% (>30%) total motility were considered as having good semen freezability (GSF), whereas boars with motility less than 30% (<30%) were considered as having poor semen freezability (PSF). Based on post-thaw sperm analysis, the semen of 21 boars was classified as having GSF, whereas the semen of 19 boars was classified as having PSF (Table 1). Besides post-thaw motility, membrane integrity of FT spermatozoa was significantly higher ($p < 0.05$) in boars of the GSF group compared with those of the PSF group (Table 1).

Table 1. Fresh, pre-freeze, and post-thaw quality of boar semen. Data are presented as the mean ± SEM of 21 and 19 boars with good and poor semen freezability (GSF and PSF, respectively).

Sperm Parameters	Fresh, Pre-Freeze Semen	Post-Thaw Semen		Total
		GSF	PSF	GSF + PSF
Motility (%)	75.78 ± 0.28	42.96 ± 1.63 [a]	26.85 ± 1.28 [b]	35.31 ± 1.65
MMP (%)	87.83 ± 2.64	48.82 ± 1.04 [a]	31.55 ± 1.55 [b]	40.62 ± 1.64
PMI (%)	87.43 ± 1.22	49.91 ± 0.84 [a]	34.55 ± 1.62 [b]	42.64 ± 1.51
NAR acrosome integrity (%)	91.62 ± 1.07	53.93 ± 0.60 [a]	40.85 ± 0.86 [b]	47.72 ± 1.38
DNA fragmentation (%)	2.97 ± 0.13	7.85 ± 0.45 [a]	10.56 ± 0.83 [b]	9.16 ± 0.50
LPO (nMol MDA/10^9spz/h)	21.34 ± 0.68	27.37 ± 0.47 [a]	31.55 ± 0.48 [b]	29.36 ± 0.45

Values with different letters (a and b) are significantly differed ($p < 0.05$); MMP—mitochondrial membrane potential; PMI—plasma membrane integrity; NAR—normal apical ridge; LPO—lipid peroxidation; MDA—malondialdehyde.

2.2. RNA-Seq Mapping and Variant Calling

Clean reads that were mapped to the reference genome ranged from 38,651,853 to 58,239,476, corresponding to about 72.8% uniquely mapped reads (range, 69.8% to 76.7%). The results of the mapping procedure were merged into a single binary alignment map (BAM) file for each boar. The fastq sequence dataset of each library is accessible in the NCBI-SRA database (Bioproject: PRJNA415904; accession number: SRP121647).

Using rMATS-DVR software, in conjunction with the genome analysis tool kit (GATK), a total of 1,389,568 variants were identified in the sperm RNA-Seq datasets of the six boars. Analysis of the filtered/trimmed data base single nucleotide polymorphisms (dbSNPs) resulted in an average of 599,428 raw SNPs, corresponding to approximately 88,340 SNPs (≥10% reads ≥90%) and 82,356 SNPs (≥10% reads = 100%), as shown in Table 2. Following stringent filtering analysis of the dbSNPs, 1371 SNP variants were annotated (Supplementary Table S1), of which about 67% putative polymorphisms (919/1371) were located at the 3′-untranslated regions (3′-UTRs) (Table 3). Furthermore, variant calling detected approximately 5.4% missense polymorphism (74/1371) in genes expressed in boar spermatozoa (Table 3).

Table 2. Analysis of filtered and trimmed dbSNPs in boar spermatozoa using variant calling of RNA-Seq datasets.

Sample ID	Raw SNV	≥10% Reads ≥90%	% Raw Reads	≥10 Reads = 100%	Raw dbSNPs	≥10% Reads ≥90%	% Raw Reads	≥10 Reads = 100%
G01	941,355	80,714	8.6%	66,625	632,893	68,577	10.8%	57,182
G09	873,327	78,084	8.9%	73,323	583,610	66,131	11.3%	62,796
G17	941,859	144,415	15.3%	135,223	618,586	122,917	19.9%	116,302
P30	866,451	100,806	11.6%	94,399	575,549	84,148	14.6%	79,695
P38	890,984	110,574	12.4%	103,347	591,527	94,160	15.9%	88,990
P39	898,293	110,753	12.3%	103,664	594,402	94,107	15.8%	89,169

dbSNP—data base single nucleotide polymorphism; SNV—single nucleotide variant.

Table 3. Putative SNPs detected in boar spermatozoa.

SNP Effect	SNP Count	Percent (%)
3 prime UTR variant	919	67.031
5 prime UTR variant	120	8.752
Synonymous variant	100	7.294
Missense variant	74	5.398
Non coding transcript exon variant	57	4.158
Splice region variant	4	0.292
Intron variant, non-coding transcript variant	3	0.219
Stop gained	2	0.145
Others	4	0.292
Unknown	88	6.419

2.3. SNP Abundance in Functional Categories of Candidate Genes (CGs)

A total of approximately 180 CG genes were enriched in at least 27 pathways, and were mainly distributed in the inflammatory-related pathways followed by the glutamatergic synapse and signaling pathways (Supplementary Table S2). Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway showed a high number of the CG genes were enriched in the PI3K-Akt signaling and metabolic pathways (Supplementary Table S2).

Enrichment analysis showed the gene ontology (GO) terms of the CGs (Figure 1, Supplementary Table S3). Generally, the CGs were associated with various functional categories, such as biological process categories (Figure 1A), molecular function categories (Figure 1B), and cellular component categories (Figure 1C). The more prominent enriched terms of the GO biological process categories included "cellular response to stimulus" (*APPL1, TXNIP,* and *OXSR1*) and "cell communication" (*MYO3B*, *EXOC4,* and *KIF1B*), as shown in (Figure 1A). The GO molecular function categories were mainly associated with "hydrolase activity" (*PARG*, *GBA3,* and *GM2A*) and "catalytic activity, acting on a protein" (*HARS2*, *PLBD1,* and *SARS*) (Figure 1B). In addition, the GO cellular component categories included enriched terms associated with "cell" (*CFAP52*, *ANKRD50,* and *SKAP2*) and "intracellular" (*EVA1A*, *TRIM9,* and *PRICKLE1*) (Figure 1C).

The selected CGs for KASP genotyping markers are highly associated with various reproductive processes and sperm functions (Supplementary Table S4). Analysis of the reproduction quantitative traits (QTLs) of the selected CGs showed that age at puberty, corpus luteum number, litter size, and number of stillborns were commonly detected among the pig reproductive traits, according to the Animal Genome pigQTLdb (https://www.animalgenome.org/cgi-bin/QTLdb/SS/index).

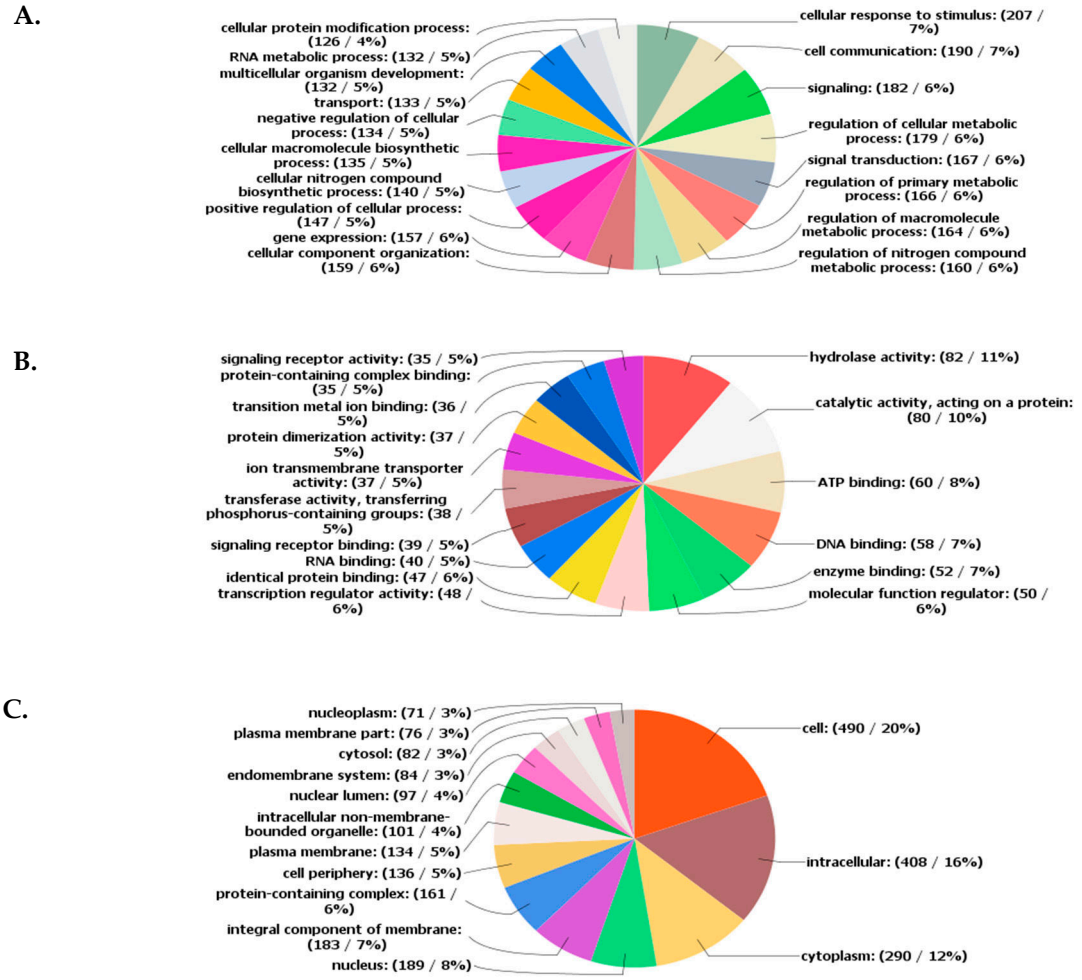


Figure 1. Gene ontology (GO) of enriched terms associated with candidate genes (CGs) in boar spermatozoa. **A**. Biological process categories; **B**. molecular function categories; **C**. cellular component categories. The values in the parenthesis indicate the number of inputs and the percentage of CGs enriched to the category.

2.4. KASP Genotyping and Validation

Following KASP genotyping, the allele call rate was 92%, and the genotyping of three SNP markers—*AHI1*, *GLMN*, and *IFNAR2*—was unsuccessful, probably due to poor design of the primers. It was found that *MY3OB* was monomorphic, whereas three other SNP markers—*CDK17*, *CLNK*, and *CRISP2*—showed an excess of homozygotes or heterozygotes in either freezability group. These SNPs were removed from further analysis. Among the SNPs analyzed in this study, the χ2-test and probability estimates showed that most of the SNP loci were within in the Hardy–Weinberg equilibrium (HWE) expectations (Table 4). Noticeable significant departures ($p < 0.05$) from HWE were common in either the GSF group or PSF group, or in both freezability groups, such as at *A2M* g.62485457G > A (rs339026428) and *COMMD2* g.189987893G > A (rs318435440) loci (Table 4).

There were variations in the frequency distributions of the alleles of the CGs (Table 4). Irrespective of the freezability group, higher frequency of allele G was observed in *CYP7B1* (0.90) gene locus (Table 4A), whereas allele A was more frequent in *CFAP52* (0.72) gene locus (Table 4B). Higher frequency of allele C was observed in *EML6* (0.72) gene locus (Table 4C), whereas higher frequency distributions of allele T were observed in *ROBO1* (0.87) gene locus (Table 4F).

Genotyping cluster plots of a few genes with polymorphism used for KASP genotyping assay are shown in Figure 2A–F (*APPL1*, *OXSR1*, *FBXO16*, *RAB3C*, *PLBD1*, and *SARS*). Samples shown in pink dots were not assigned to the cluster.

Table 4. Genic differentiation, frequencies of single nucleotide polymorphism (SNP) alleles, and Hardy–Weinberg equilibrium (HWE) with respective *p*-value, chi-square (χ2), and probability. GSF—good semen freezability; PSF—poor semen freezability.

Table 4**A**

SNP ID	Locus	Freezability Groups	Allele Counts		Total	Allele Frequencies		*p*-value	HWE		
			A	G		A	G		*p*-value	χ2	Probability
unknown	*APPL1*	GSF	19	23	42	0.452	0.548	1.00000	1.0000	5.8659	0.2094
		PSF	15	19	34	0.441	0.559		0.0532		
		Total	34	42	76	0.447	0.553				
rs338842672	*CYP7B1*	GSF	5	35	40	0.125	0.875	0.71180	0.0105	9.1044	0.0585
		PSF	3	35	38	0.079	0.921		1.0000		
		Total	8	70	78	0.103	0.897				
rs345056502	*EML5*	GSF	12	30	42	0.286	0.714	0.06221	0.1283	6.1640	0.1872
		PSF	17	17	34	0.500	0.500		0.3575		
		Total	29	47	76	0.382	0.618				
unknown	*LPAR1*	GSF	26	14	40	0.650	0.350	0.62845	0.6347	2.0228	0.7316
		PSF	24	10	34	0.706	0.294		0.5731		
		Total	50	24	74	0.676	0.324				
rs81210636	*RAB3C*	GSF	11	31	42	0.262	0.738	0.10038	1.0000	7.5732	0.1085
		PSF	17	21	38	0.447	0.553		0.0227		
		Total	38	52	80	0.350	0.650				
rs344846507	*SARS*	GSF	19	21	40	0.475	0.525	0.50110	1.0000	8.8484	0.0650
		PSF	15	23	38	0.395	0.605		0.0120		
		Total	34	44	78	0.436	0.564				
rs340075321	*TXNIP*	GSF	12	30	42	0.286	0.714	0.10631	0.1301	14.0970	0.0070
		PSF	18	20	38	0.474	0.526		0.0067		
		Total	30	50	80	0.375	0.625				

Table 4. *Cont.*

Table 4**B**

SNP ID	Locus	Freezability Groups	Allele Counts		Total	Allele Frequencies		*p*-value	HWE		
			G	A		G	A		*p*-value	χ2	Probability
rs339379734	*OXSR1*	GSF	18	24	42	0.429	0.571	0.48138	0.3775	8.4171	0.0774
		PSF	17	15	32	0.531	0.469		0.0394		
		Total	35	39	74	0.473	0.527				
rs339026428	*A2M*	GSF	8	34	42	0.190	0.810	0.36658	0.0001	26.9432	0.0000
		PSF	2	36	38	0.053	0.947		0.0271		
		Total	10	70	80	0.125	0.875				
rs81210697	*ANKRD42*	GSF	16	26	42	0.381	0.619	0.49879	0.6413	18.6650	0.0009
		PSF	20	18	38	0.526	0.474		0.0015		
		Total	36	44	80	0.450	0.550				
rs332902509	*CCDC149*	GSF	17	25	42	0.405	0.595	0.48804	0.3681	7.5901	0.1078
		PSF	17	17	34	0.500	0.500		0.0611		
		Total	34	42	76	0.447	0.553				
unknown	*CFAP52*	GSF	28	12	40	0.700	0.300	0.79820	0.6178	3.5245	0.4742
		PSF	25	9	34	0.735	0.265		0.2779		
		Total	53	21	74	0.716	0.284				
rs318435440	*COMMD2*	GSF	19	21	40	0.475	0.525	0.64518	0.0050	19.7578	0.0006
		PSF	14	20	34	0.412	0.588		0.0102		
		Total	33	41	74	0.446	0.554				
rs341614458	*FBXO16*	GSF	16	24	40	0.400	0.600	0.16668	1.0000	0.9449	0.9180
		PSF	21	15	36	0.583	0.417		0.6235		
		Total	37	39	76	0.487	0.513				
rs340643892	*MAP3K20*	GSF	11	27	38	0.289	0.711	1.00000	0.2531	5.3360	0.2545
		PSF	9	25	34	0.265	0.735		0.2741		
		Total	20	52	72	0.278	0.722				
rs319208708	*WRN*	GSF	9	29	38	0.237	0.763	0.76470	1.0000	0.0000	1.0000
		PSF	5	21	26	0.192	0.808		1.0000		
		Total	14	50	64	0.212	0.788				

Table 4. *Cont.*

Table 4**C**										
SNP ID	**Locus**	**Freezability Groups**	**Allele Counts**		**Total**	**Allele Frequencies**		***p*-value**	**HWE**		
			A	**C**		**A**	**C**		***p*-value**	**χ2**	**Probability**
rs325939188	*CLEC7A*	GSF	15	27	42	0.357	0.643	1.00000	0.6647	3.2122	0.5230
		PSF	13	21	34	0.382	0.618		0.3019		
		Total	28	48	76	0.368	0.632				
rs322659685	*EML6*	GSF	10	30	40	0.250	0.750	0.60588	1.0000	4.4370	0.3501
		PSF	11	23	34	0.324	0.676		0.3501		
		Total	21	53	73	0.284	0.716				
			C	**A**		**C**	**A**				
rs324930519	*ABCB11*	GSF	16	24	40	0.400	0.600	0.62897	0.0005	15.2834	0.0041
		PSF	11	23	34	0.324	0.676		1.0000		
		Total	27	47	74	0.635	0.365				
rs343122214	*SMS*	GSF	12	26	38	0.316	0.684	0.33352	0.0015	13.8162	0.0079
		PSF	16	20	36	0.444	0.556		0.6577		
		Total	28	46	74	0.378	0.622				

Table 4. *Cont.*

Table 4**D**										
SNP ID	***Locus***	**Freezability Group**	**Allele Counts**		**Allele Frequencies**		**Total**	***p*-value**	**HWE**		
			C	**G**	**C**	**G**			***p*-value**	**χ2**	**Probability**
rs80954196	*TMEM177*	GSF	21	17	38	0.553	0.447	0.81666	0.0001	23.0661	0.0001
		PSF	18	18	36	0.500	0.500		0.1750		
		Total	39	35	74	0.527	0.473				
			G	**C**		**G**	**C**				
rs339836492	*MS4A2*	GSF	10	30	40	0.250	0.750	0.32355	0.2784	9.0353	0.0602
		PSF	13	23	36	0.361	0.639		0.0392		
		Total	23	53	76	0.303	0.697				

Table 4. *Cont.*

Table 4**E**											
SNP ID	**Locus**	**Freezability Groups**	**Allele Counts**		**Total**	**Allele Frequencies**		***p*-value**	**HWE**		
			C	**T**		**C**	**T**		***p*-value**	**χ2**	**Probability**
rs694366781	*PRICKLE1*	GSF	15	27	42	0.357	0.643	1.00000	1.0000	2.2918	0.6823
		PSF	12	20	32	0.375	0.625		0.3179		
		Total	27	47	74	0.365	0.635				
rs337913978	*SCLT1*	GSF	9	33	42	0.214	0.786	0.77416	0.5322	1.2614	0.8679
		PSF	6	30	36	0.167	0.833		1.0000		
		Total	15	63	78	0.192	0.808				
			T	**C**		**T**	**C**				
rs334625232	*ACSL4*	GSF	5	37	42	0.119	0.881	0.36037	1.0000	0.0000	1.0000
		PSF	8	28	36	0.222	0.778		1.0000		
		Total	13	65	78	0.167	0.833				
rs328079913	*ATP5F1A*	GSF	22	20	42	0.524	0.476	0.06697	0.0000	>43.1295	<0.0000
		PSF	28	10	38	0.737	0.263		0.0036		
		Total	50	30	80	0.625	0.375				
rs335938037	*HSPA13*	GSF	11	19	30	0.367	0.633	0.57756	0.2960	3.7925	0.4348
		PSF	8	22	30	0.267	0.733		0.5072		
		Total	19	41	60	0.317	0.683				
rs81217594	*PAM*	GSF	11	29	40	0.275	0.725	0.77866	0.2555	2.7292	0.6041
		PSF	6	20	26	0.231	0.769		1.0000		
		Total	17	49	66	0.258	0.742				
unknown	*RIOX2*	GSF	9	29	38	0.237	0.763	0.57428	1.0000	0.0000	1.0000
		PSF	8	18	26	0.308	0.692		1.0000		
		Total	17	47	64	0.266	0.734				
rs336351767	*SKAP2*	GSF	16	26	42	0.381	0.619	0.21618	0.6422	2.1935	0.7002
		PSF	8	26	34	0.235	0.765		0.5200		
		Total	24	52	76	0.316	0.684				

Table 4. *Cont.*

Table 4**F**										
SNP ID	**Locus**	**Freezability group**	**Allele counts**		**Total**	**Allele frequencies**		***p*-value**	**HWE**		
			A	**T**		**A**	**T**		***p*-value**	**χ2**	**Probability**
rs331568674	*ROBO1*	GSF	5	37	42	0.119	0.881	0.74597	1.0000	0.0000	1.0000
		PSF	5	29	34	0.147	0.853		1.0000		
		Total	10	66	76	0.132	0.868				
			T	**A**		**T**	**A**				
rs321497623	*PLBD1*	GSF	14	28	42	0.333	0.667	0.16654	0.0487	6.0432	0.1959
		PSF	18	18	36	0.500	0.500		1.0000		
		Total	32	46	78	0.410	0.590				

Table 4. *Cont.*

Table 4**G**										
SNP ID	**Locus**	**Freezability group**	**Allele counts**		**Total**	**Allele frequencies**		***p*-value**	**HWE**		
			G	**T**		**G**	**T**		***p*-value**	**χ2**	**Probability**
rs336003721	*HARS2*	GSF	8	32	40	0.200	0.800	0.78401	0.5482	2.5152	0.6419
		PSF	8	26	34	0.235	0.765		0.5187		
		Total	16	58	74	0.216	0.784				

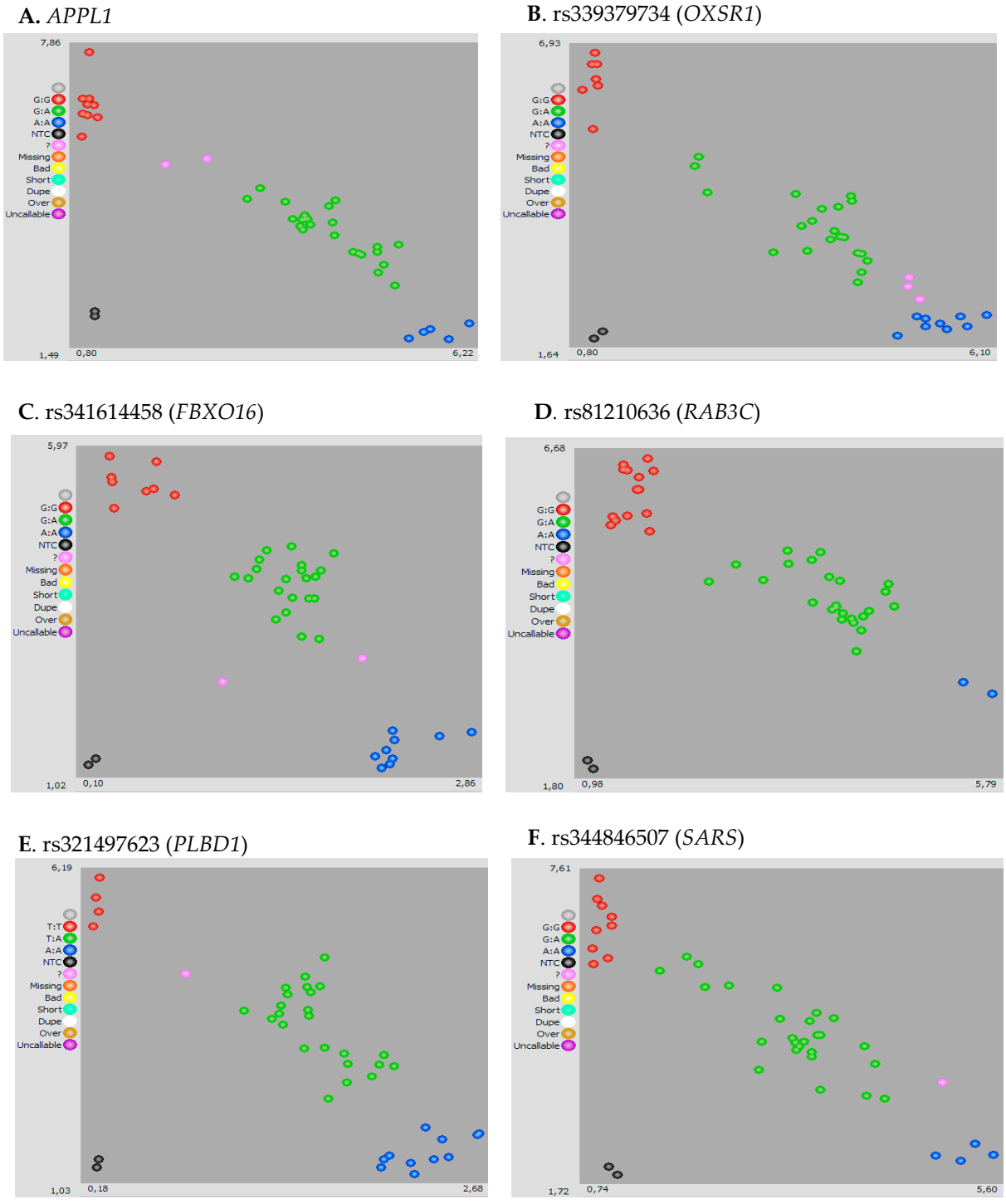


Figure 2. Genotyping cluster plots of **A**. *APPL1*, **B**. *OXSR1*, **C**. *FBXO16*, **D**. *RAB3C*, **E**. *PLBD1*, and **F**. *SARS* genes using the Kompetitive Allele Specific PCR (KASP) assay. Samples shown in pink were considered questionable genotypes as they were not assigned to the cluster. NTC, non-template control. Visualization of the genotype data was performed with the SNPViewer software (http://results.lgcgenomics.com/software/snpviewer).

2.5. Association Analysis of SNPs with Post-Thaw Semen Quality

ANOVA analysis showed that polymorphisms in *APPL1* ($p < 0.017$), *CYP7B1* ($p < 0.031$), *RAB3C* ($p < 0.043$), *OXSR1* ($p < 0.047$), *FBXO16* ($p < 0.045$), and *PLBD1* ($p < 0.046$) genes had significant effects on post-thaw sperm motility. Polymorphisms in *APPL1* and *PLBD1* genes markedly affected post-thaw

PMI ($p < 0.048$ and $p < 0.025$, respectively) and NAR acrosome integrity ($p < 0.016$ and $p < 0.018$, respectively). Also, polymorphism in *PLBD1* gene affected post-thaw MMP and LPO ($p < 0.018$ and $p < 0.043$, respectively). It was observed that polymorphisms in *SARS* gene had marked effects on post-thaw MMP ($p < 0.046$) and NAR acrosome integrity ($p < 0.001$), while *EML5* polymorphism affected post-thaw DNA integrity ($p < 0.015$), LPO ($p < 0.035$), and NAR acrosome integrity ($p < 0.048$). Furthermore, polymorphisms in *RAB3C* and *PRICKLE1* genes had marked effects on post-thaw NAR acrosome integrity ($p < 0.041$ and $p < 0.040$, respectively) and LPO ($p < 0.037$ and $p < 0.013$, respectively). In addition, polymorphisms in *FBXO16* gene affected post-thaw MMP ($p < 0.048$).

Association analysis of post-thaw semen quality showed that motility was lower ($p < 0.05$) for AG genotype of *APPL1* gene (a newly predicted SNP) (Figure 3A). Likewise, post-thaw motility was lower ($p < 0.05$) for AG genotype (AG vs. GG) at rs338842672 (*CYP7B1* gene) and rs81210636 (*RAB3C* gene) (Figure 3B,C, respectively). Furthermore, post-thaw motility was lower ($p < 0.05$) for the heterozygous genotype at rs339379734 (*OXSR1* gene) compared with AA genotype (Figure 3D), and for GG genotype at rs341614458 (*FBXO16* gene), as shown in Figure 3E. As regards *PLBD1* gene polymorphism, post-thaw motility was the lowest for TT genotype at rs321497623 (Figure 3F).

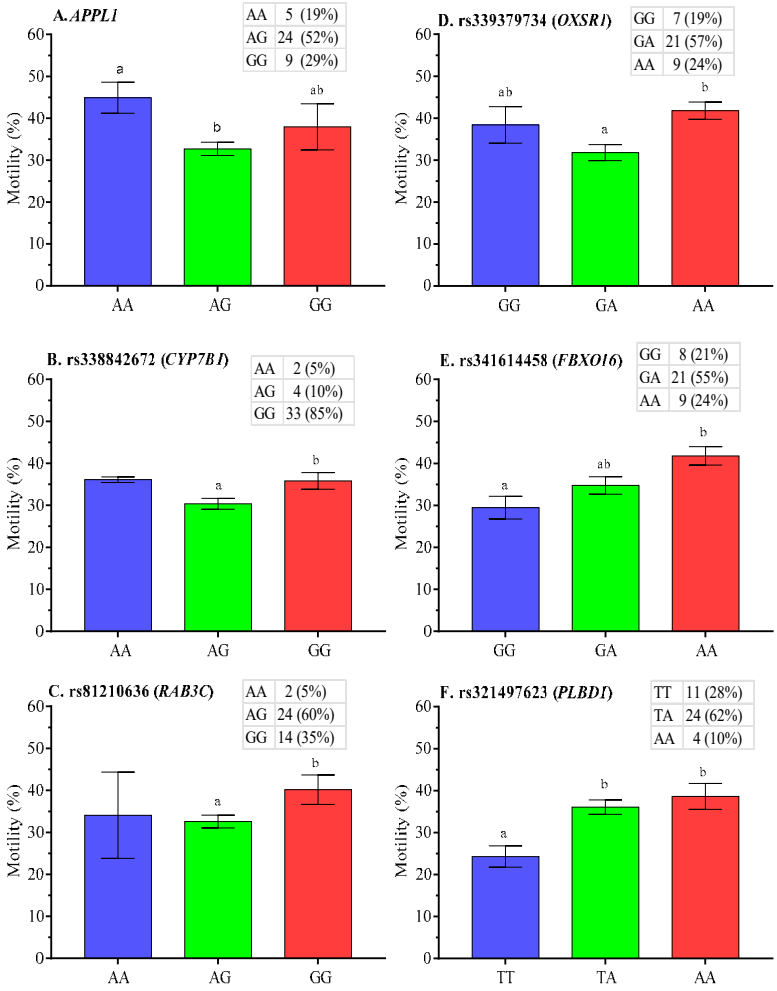


Figure 3. Effect of polymorphisms in **A**. *APPL1*, **B**. *CYP7B1*, **C**. *RAB3C*, **D**. *OXSR1*, **E**. *FBXO16*, and **F**. *PLBD1* genes on post-thaw motility of boar spermatozoa. Data are presented as the mean ± SEM; [a,b] Differences between genotypes were significantly differed at $p < 0.05$.

Significantly lower ($p < 0.05$) post-thaw MMP (Figure 4A), PMI (Figure 4B), and acrosome integrity (Figure 4C) were concurrent with higher post-thaw LPO for TT genotype at rs321497623 (Figure 4D).

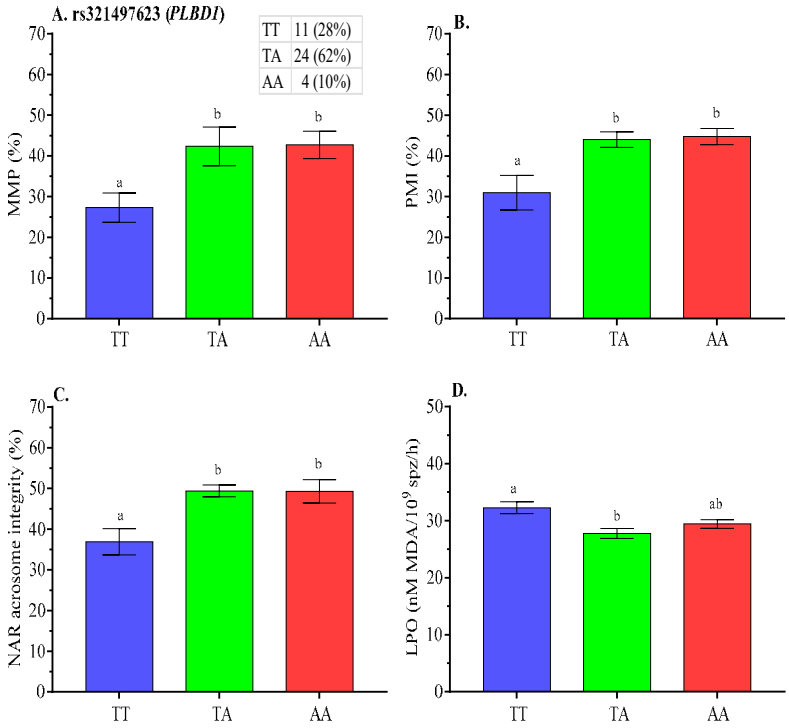


Figure 4. Effect of *PLBD1* gene polymorphism on post-thaw **A**. MMP, **B**. PMI, **C**. NAR acrosome integrity, and **D**. LPO of boar spermatozoa. Data are presented as the mean ± SEM. [a,b] Differences between genotypes were significantly differed at $p < 0.05$.

Boars with AG genotype of *APPL1* gene had significantly lower ($p < 0.05$) post-thaw PMI and NAR acrosome integrity (Figure 5A,B, respectively), while lower post-thaw NAR acrosome integrity and higher LPO were observed for AA genotype at rs345056502 (*EML5* gene) (Figure 5C,D, respectively). Furthermore, post-thaw DNA damage was highest ($p < 0.05$) for AA genotype at rs345056502 (Figure 5E), while lower post-thaw MMP was observed for GG genotype at rs341614458 (Figure 5F).

Post-thaw analysis showed that boars with AG genotype had lower ($p < 0.05$) MMP and acrosome integrity at rs344846507 (*SARS* gene) (Figure 6A,B, respectively). It was found that lower ($p < 0.05$) post-thaw NAR acrosome integrity was concomitant with higher LPO for genotype AG (AG vs. GG) at rs81210636 (Figure 6C,D, respectively). Higher post-thaw acrosome damage and LPO were associated with CT or TT genotype at rs694366781 (*PRICKLE1* gene) (Figure 6E,F, respectively).

ANOVA analysis revealed that freezability (F) and SNP genotype (G) and their interactions (F × G) significantly affected ($p < 0.05$) post-thaw semen quality (Table 5). Significant F × G interactions with post-thaw motility and PMI were observed for rs337913978 (*SCLT1* gene, Table 5A). Also, freezability ($p < 0.001$) and F × G interaction ($p < 0.010$) with post-thaw NAR acrosome integrity were observed for rs340643892 (*MAP3K20* gene, Table 5B). Post-thaw LPO and DNA fragmentation showed significant SNP effects and F × G interaction for rs339836492 (*MS4A2* gene) and rs331568674 (*ROBO1* gene), respectively (Table 5C,D, respectively).

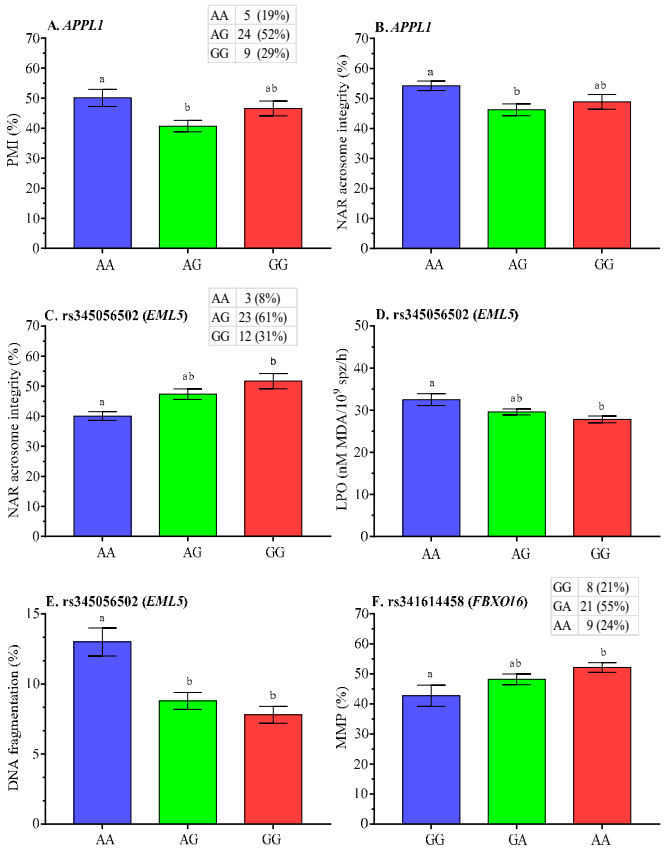


Figure 5. Effect of polymorphisms in **A–B**. *APPL1*, **C–E**. *EML5*, and **F**. *FBXO16* genes on post-thaw membrane integrity of boar spermatozoa. Data are presented as the mean ± SEM. [a,b] Differences between genotypes were significantly different at $p < 0.05$.

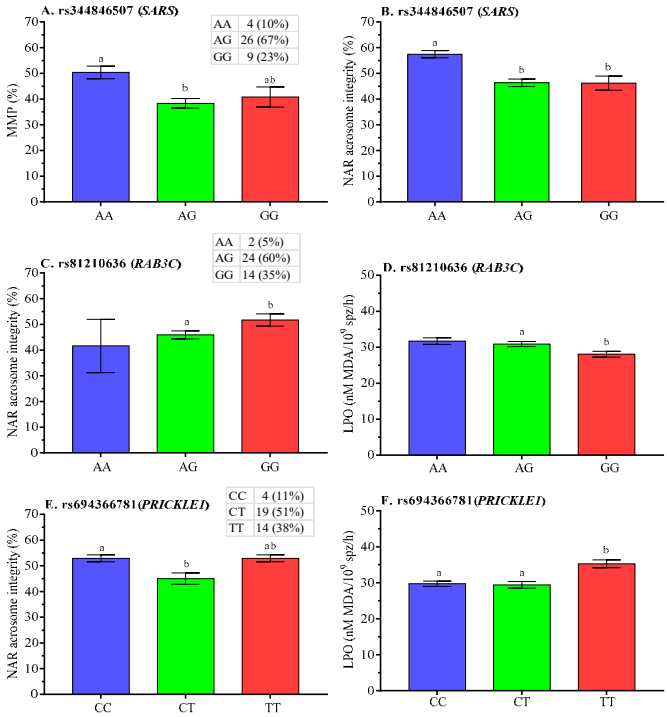


Figure 6. Effect of polymorphisms in **A–B**. *SARS*, **C–D**. *RAB3C*, and **E–F**. *PRICKLE1* genes on post-thaw membrane integrity of boar spermatozoa. Data are presented as the mean ± SEM. [a,b] Differences between genotypes were significantly different at $p < 0.05$.

Table 5. ANOVA analysis showing the effects of freezability (F) and gene polymorphism (G) on post-thaw semen quality.

A. rs337913978 (*SCLT1*)	**Motility**			**Plasma Membrane Integrity (PMI)**		
	F	**G**	**F × G**	**F**	**G**	**F × G**
	p-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value
	<0.001	>0.305	<0.043	<0.001	>0.063	<0.028
B. rs340643892 (*MAP3K20*)	**NAR Acrosome Integrity**			**Lipid Peroxidation (LPO)**		
	F	**G**	**F × G**	**F**	**G**	**F × G**
	p-value	*p*-value	*p*-value	*p*-value	*p*-value	*p*-value
	<0.001	>0.146	<0.010	<0.001	<0.022	>0.981
C. rs339836492 (*MS4A2*)	**Lipid Peroxidation (LPO)**					
	F	**G**	**F × G**			
	p-value	*p*-value	*p*-value			
	<0.001	<0.039	>0.302			
D. rs331568674 (*ROBO1*)	**DNA Fragmentation**					
	F	**G**	**F × G**			
	p-value	*p*-value	*p*-value			
	<0.030	>0.800	<0.047			

Significantly differed at $p < 0.05$; NAR—normal apical ridge.

Association analysis of post-thaw sperm quality showed that PSF boars with CT and TT genotypes at rs337913978 (*SCLT1* gene) exhibited lower ($p < 0.05$) motility and PMI (Figure 7A,B, respectively). Significantly ($p < 0.05$) lower proportions of FT spermatozoa with acrosome integrity were concomitant with higher LPO for AA genotype at rs340643892 (*MAP3K20* gene) in boars from the PSF group (Figure 7C,D, respectively). Post-thaw LPO was higher ($p < 0.05$) at rs339836492 (*MS4A2* gene) (Figure 7E), while post-thaw DNA damage was greater ($p < 0.05$) for TT genotype at rs331568674 (*ROBO1* gene) in boars of the PSF group (Figure 7F).

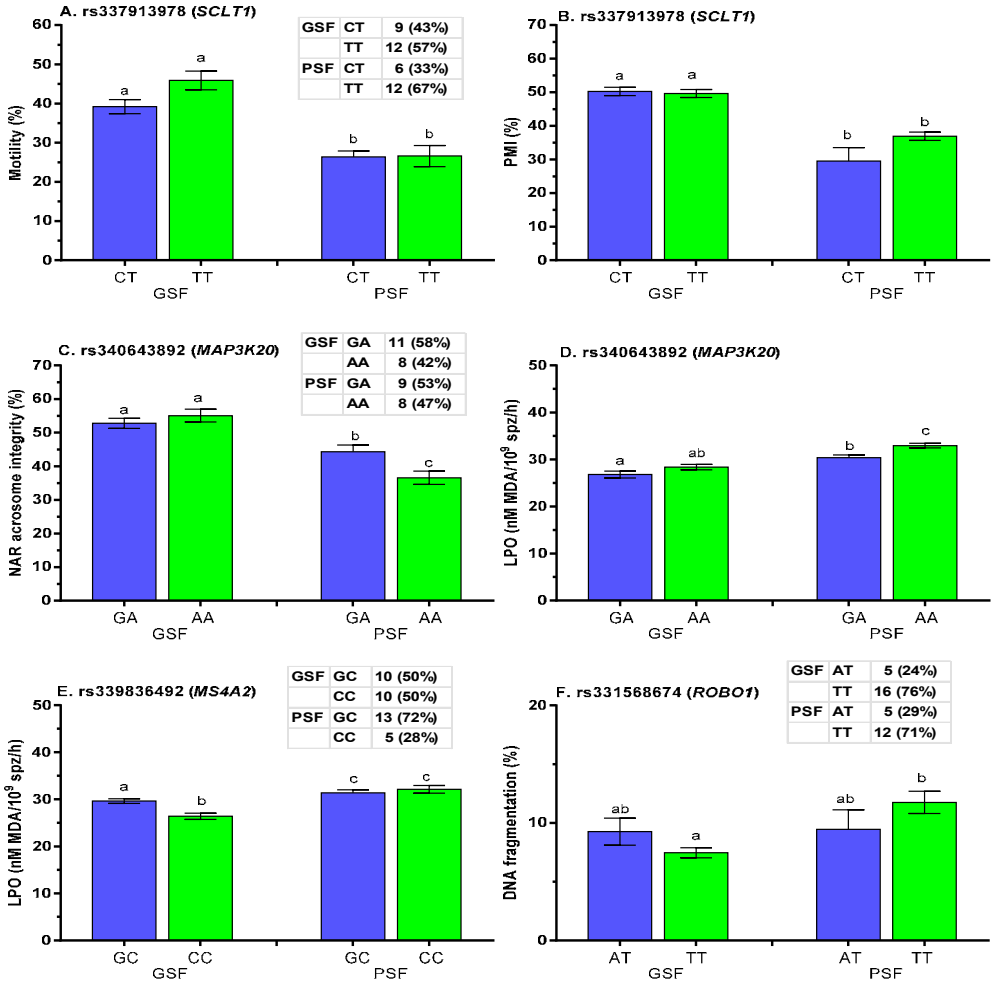


Figure 7. Effect of freezability and gene polymorphisms in **A–B**. *SCLTL1*, **C–D**. *MAP3K20*, **E**. *MS4A2*, and **F**. *ROBO1* genes on post-thaw quality of boar semen. Data are presented as the mean ± SEM. [a,b] Differences between genotypes were significantly different at $p < 0.05$; GSF—good semen freezability; PSF—poor semen freezability.

3. Discussion

3.1. SNP Functional Classes and Validation

In the present study, among the RNA-Seq SNPs (Supplementary Table S1), approximately 82% of the putative polymorphisms in boar spermatozoa had a corresponding dbSNP entry. In a previously published paper, about 88–91% of the detected SNPs in the testis tissue of boars had a dbSNP entry, and a large number of SNPs were at the 3′-UTRs, the untranslated region of noncoding mRNAs [7]. In this study, we found that about 67% of putative polymorphisms were at the 3′-UTR (Table 3), and the impact of most of these polymorphisms on sperm function is not fully understood. Studies have reported that the 3′-UTR plays an important role in the translation efficiency and stability of mRNA [17,18]. It has been reported that UTRs have strong impacts on post-transcriptional regulation of gene expression, suggesting that dysfunction in the UTRs might have a significant effect on gene expression and the associated cellular viability, growth, and development [17,18]. Among the UTR variants at the 3′-UTR, two SNP polymorphisms, rs339379734 (*OXSR1*) and rs337913978 (*SCLT1*), showed significant associations with post-thaw semen quality. Evidence has indicated that SNPs at the 3′-UTR of the targeted mRNA have been the principal elements of microRNA adhesion, and they are associated with semen quality [19]. We suggest that the 3′-UTR SNPs, detected in this study, might play a significant role at the transcription level. Furthermore, most of the coding sequences detected

in spermatozoa were synonymous variants, which do not affect protein sequence, but could be in linkage disequilibrium to other causative mutations [7,12] and could have functional effects on mRNA stability and, ultimately, the phenotype traits [20]. In the present study, eight SNP markers that showed significant associations with post-thaw semen quality were synonymous variants (in *CYP7B1*, *FBXO16*, *RAB3C*, *SARS*, *MAP3K20*, *PLBD1*, *EML5*, and *PROCKLE1* genes), which could be linked to causative mutations with functional effects on the reproductive traits in the pig. Furthermore, two missense polymorphisms in *APPL1* and *MS4A2* genes showed significant associations with post-thaw semen quality. Missense variants affect amino acid translation, protein structure and function, and several missense polymorphisms have been shown to affect enzyme activity [21]. It is reasonable to deduce that the missense polymorphisms in *APPL1* (a novel predicted SNP) and *MS4A2* (rs339836492) genes could have a marked effect on their protein expression levels, which could consequently compromise the sperm response to the freezing–thawing conditions.

Quality assessment of a panel of SNP markers was performed by KASP genotyping assay. It is noteworthy that KASP is a novel competitive allele specific PCR, which is mainly based on the amplification of DNA with a thermal cycler using allele specific primers [22]. Due to its low cost and high efficiency, the KASP genotyping assay has been widely used in SNP genotyping studies related to molecular marker-assisted selection breeding [23]. In this study, the genotype frequencies were consistent with the HWE assumptions for most of the analyzed loci. Screening of SNPs for HWE departure is frequently used when performing association studies [24]. There is little consensus on the appropriate *p*-value threshold for the identification of SNPs that violate the HWE assumption in association studies [25]. In the current study, it is unclear why HWE departure was more common in gene loci of individuals from the PSF group. It seems likely that the marked departure from the HWE could be related to long-term artificial selection and breeding of pigs. Several authors suggested that long-term selective breeding for production traits, not associated with reproductive traits, could be attributed to the marked differences in the genotype frequencies [14,26]. Moreover, it has been confirmed that differences in the allele frequencies in AI boars highly selected for a number of traits that are responsible for HWE departure [26]. It is likely that HWE departure is not necessarily caused by the selection of the analyzed traits, but rather other traits under the control of the selected genes or closely related genes [26]. We suggest that further studies are needed to find out whether such a phenomenon is a common feature of polymorphisms in genes expressed in poor freezability ejaculates.

3.2. SNP Associations with Post-Thaw Semen Quality

Evidence has shown that the evaluation of various sperm traits rather than a single trait analysis provides a better fertility prediction of FT semen [27–29]. Several sperm attributes have been used to assess the post-thaw quality of boar semen, and among these sperm motility is widely used in semen quality assessment because it correlates with fertility outcomes [30,31]. Sperm motility is assessed subjectively or with the computer-assisted semen analysis (CASA) system; however, subjective motility assessment has been shown to provide reliable estimates in association studies [26,32,33]. In the current study, several sperm attributes have been used to monitor the associations of gene polymorphisms with post-thaw quality of boar semen.

The distribution of genotype frequencies differs among the genotype groups, with respect to polymorphisms in the genes related to GO term, "response to stress", such as *APPL1*, *OXSR1* (rs339379734), *MAP3K20* (rs340643892), and *MS4A2* (rs339836492). Since stress plays an important role in semen cryopreservation [34], the function and localization of these genes are critical in understanding their impact on post-thaw semen quality. In general, the heterogeneous genotype of the *APPL1* gene polymorphism has been associated with reduced post-thaw semen quality. It is noteworthy that the *APPL1* polymorphism is a newly predicted SNP variant detected in boar spermatozoa, and it is very difficult to make comparisons with other studies. The *APPL1* gene is related to several GO terms, such as "signal transduction", "cell motility", and "transmembrane transport", suggesting its significant relevance in sperm function. Accumulating evidence has shown that *APPL1* regulates the function of the

adiponectin receptor (AdipoR1), which is implicated in various biological processes in the reproductive system [35,36]. Adiponectin is abundantly found in the tail region of bull spermatozoa, whereas AdipoR1 has been detected mainly in the equatorial and acrosome regions of sperm [35]. Furthermore, a direct role of the AdipoR1 receptor has been suggested in sperm capacitation [36], and adiponectin and its receptors have been associated with motility parameters of ram spermatozoa [37]. These findings reaffirm the important modulating function of the *APPL1* gene on the action of the AdipoR1 receptor in regulating sperm motility. Consistent with this notion, it seems that polymorphisms in the *APPL1* gene might be a contributing factor to compromised function of FT spermatozoa.

The *OXSR1* gene regulates downstream kinases in response to environmental stress, and it plays a role in the Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) in the testis [38]. Moreover, *OXSR1* is one of the upstream phosphorylators of the NKCC1, which is activated through a phosphorylation-dependent mechanism and is implicated in sperm–oocyte fertilization events [38]. It seems, therefore, that an *OXSR1* gene polymorphism could reduce the abundance of phosphorylated-NKCC1, resulting in reduced sperm motility and compromised IVF potential [38]. Association analysis shows that heterogeneous genotype of *OXSR1* gene significantly increases the sperm susceptibility to reduced post-thaw motility. Hence, it seems likely that the *OXSR1* gene polymorphism compromises the sperm's ability to confer protection against oxidative stress, particularly during the freezing–thawing process. Cryo-induced stress increases the sperm susceptibility to reactive oxygen species (ROS)-related damage, such as mitochondrial dysfunction, peroxidation of sperm membrane lipids, and DNA damage, resulting in compromised fertility of FT spermatozoa [5,29,39]. Furthermore, it seems that the AA genotype at rs340643892 (*MAP3K20* gene) might predispose spermatozoa to increased LPO and acrosome damage after freezing–thawing. It should be noted that MAPK is a serine/threonine kinase, which is implicated in the activation of gene transcription and expression [40]. Moreover, the MAPK cascade elements have been detected in the flagellum of human spermatozoa and are involved in the regulation of capacitation and the acrosome reaction processes [40]. Analysis shows that GC and CC genotypes at rs339836492 (*MS4A2* gene) were associated with increased post-thaw LPO in poor freezability ejaculates, suggesting that spermatozoa from these ejaculates were more susceptible to increased damage incurred by ROS action during the freezing–thawing process. Although the functions of many of the *MS4A* proteins are currently not well defined, it was reported that the *MS4A* family member could form oligomers in sperm membranes, which might be involved in the interaction with the zona pellucida or cumulus during fertilization [41]. It is noteworthy that the *MS4A2* transcript has been detected in the sperm head [42], suggesting its relevance in sperm–oocyte interaction mechanisms [41]. Our results show that polymorphisms in *MAP3K20* and *MS4A2* genes are associated with reduced post-thaw semen quality, which was manifested mainly in increased susceptibility of FT spermatozoa to LPO, as observed in poor freezability ejaculates. Moreover, an increase in LPO of FT spermatozoa results in a substantial loss of membrane integrity and motility, and ultimately leads to reduced fertility [43]. Additional studies are required to determine the biological relevance of these gene polymorphisms in sperm functions.

Interestingly, it has been confirmed that heterozygous AG (AG vs. GG) of *CYP7B1* gene (rs338842672) is a contributing factor to reduced post-thaw motility. The *CYP7B* gene is enriched in the primary bile acid biosynthesis pathway (ssc00120) and 14 GO terms including "reproduction", "oxidoreductase activity", "cell motility", and "signal transduction", suggesting the important role of the gene in sperm functions. However, we would like to emphasize that the consequences of *CYP7B1* polymorphism on post-thaw quality of boar semen are not yet known. Similar to the *CYP7B1* gene, only two genotypes (AG vs. GG) of the *RAB3C* gene were considered in the association studies with post-thaw semen quality. In support of this notion, analysis showed that heterozygous AG at rs81210636 (*RAB3C* gene) is also a contributing factor to reduced post-thaw semen quality compared with the GG genotype. The impact of *RAB3C* gene on post-thaw semen quality is unclear. Not much information is available about the role of the *RAB3C* gene in sperm function, but earlier research reported that the *RAB3* protein could interact with a series of proteins and could be associated with

the cAMP/PKA (cAMP/protein kinase A) messenger system, in conjunction with phospholipase A_2 (PLA_2), to modulate exocytosis of the sperm acrosome [44]. Moreover, the *RAB3* gene is related to 7 GO enriched terms, such as "GTPase activity", "signal transduction", and "vesicle-mediated transport", suggesting the important role of the gene in sperm biological functions. It seems that the higher genotype frequency of heterozygous AG (60%) might predispose FT spermatozoa to compromise fertilization-related events. However, it is unclear how the *RAB3C* protein interacts with boar sperm membrane, and its role in cryo-survival has not yet been elucidated.

In our study, polymorphisms in *FBXO16* (rs341614458) and *PLBD1* (rs321497623) genes were associated with motility and membrane integrity of FT spermatozoa. It should be emphasized that there is not enough information in the literature about the specific role of these genes in the physiological functions of the sperm. It seems that the GG genotype of the *FBXO16* gene is a contributing factor for reduced post-thaw semen quality. The effect of the *FBXO16* polymorphism on sperm function is not fully known; however, members of the F-box family bind to phosphorylated proteins to promote their ubiquitination and degradation, which are required to protect and maintain sperm quality [45,46]. Evidence has shown that ubiquitin-mediated proteolysis of *FBXO* proteins is indispensable for the stability of sperm organelles [45]. Therefore, dysfunction in the ubiquitin-related pathway might have relevance in the physiological functions of the sperm. Furthermore, the effects of *PLBD1* gene polymorphism on the biological function of the sperm is still unclear, even though it has been reported that *PLBD1* is implicated in the fertilization processes [47]. Moreover, the *PLBDI* gene is related to 12 GO enriched terms, such as "lipid metabolic process", "signal transduction", and "kinase activity", suggesting the important role of the gene in various sperm biological functions. It can be suggested that an understanding of the functional significance of *FBXO16* and *PLBD*1 genes is necessary to elucidate their biological effects on sperm functions following cryopreservation.

Analysis showed that polymorphisms in the genes associated with cytoskeleton proteins, namely *EML5* (rs345056502) and *PRICKLE1* (rs694366781), were significantly correlated with reduced motility and acrosome integrity, and increased LPO of FT spermatozoa. It is noteworthy that the cytoskeleton is implicated in various cellular functions including mitosis, membrane translocations, and cellular motility [48]. The impact of polymorphisms of these genes on sperm function is not fully understood. However, our results provide evidence indicating that boars with the homologous AA genotype at rs345056502 (*EML5* gene) were more susceptible to post-thaw acrosome damage and LPO, while the homologous TT genotype of the *PRICKLE1* gene contributed to greater cryo-induced DNA damage. Moreover, microtubule-associated proteins (MAPs) in sperm cells have various functions including modulation of actin cytoskeletal function during spermatogenesis [49]. Collectively, *EML5* and *PRICKLE1* are implicated in microtubule (MT) dynamics in the testis, and mutations in their genotypes could affect the physiological and structural function of the sperm following cryopreservation. Interestingly, no association of either *EML5* or *PRICKLE1* genotypes with post-thaw motility was observed this study. This is surprising due to the critical role of MT or MAPs in the motility apparatus of spermatozoa. A possible explanation could be that the microscope assessment used in this study gives only one measured parameter of sperm motility, while the CASA system provides a variety of different motility parameters [29,50], which might be useful in association studies.

Of the gene associated with t-aminoacylation is *SARS*, polymorphism (rs344846507) in this gene was associated with mitochondrial function and acrosome integrity of FT spermatozoa. It is noteworthy that SARS is a nuclei acid binding gene related to tRNA-aminoacylation biosynthesis (ssc00970) and mitochondrial disorders. Earlier observation showed that increased abundance of *SARS* proteins in spermatozoa resulted in poor blastocyst development [51]. The finding of this study raises the possibility that the *SARS* polymorphism could have an important effect on male fertility, and this necessitates further studies. The *SCLT1* gene is also of importance, in which the frequency distributions of either CT or TT genotype at rs337913978 are associated with reduced post-thaw semen quality. Enrichment analysis showed that the *SCLT1* gene is associated with 9 GO terms, such as "microtubule organizing center" and "cilium", suggesting the critical role of the gene in sperm function. It has been

reported that the *SCLT1* gene polymorphism causes dysfunction in axoneme assembly [52], which could compromise sperm motility. Presently, no significant associations between the *SCLT1* gene and post-thaw semen quality have been reported. Another polymorphism (rs331568674) of interest was detected in the *ROBO1* gene. Our findings show that the *ROBO1* gene is enriched in the axon guidance pathway (ssc04360) and 12 GO terms involved in biological functions, such as "cell motility", "signal transduction", and "protein modification process". In a recent study, it has been reported that the axon guidance pathway has a potential biological function in boar fertility [53], and its pathway has a significant role in DNA methylation alterations in sperm cell development [54]. In the current study, it seems that the TT genotype of the *ROBO1* gene polymorphism might increase the sperm susceptibility to cryo-induced DNA fragmentation, as shown in the poor freezability ejaculates. It should be stressed that the homologous genotype was not detected in *SCLT1* (CT-TT), *MAP3K20* (GA-AA), *MS4A2* (GC-CC), and *ROBO1* (AT-TT) genes. Previous studies reported that the absence of a homologous genotype might indicate its removal through artificial selection and breeding [32,33]. Artificial selection and breeding is constantly practiced in the pig AI industry, and it is possible that such phenomenon might explain the absence of the homologous genotype in the above-mentioned gene polymorphisms. Furthermore, we are unable to explain the presence of low frequency of alleles in the homologous genotype of genes, such as *CYP7B1* and *RAB3C*. Besides intensive selective breeding, it has been suggested that some genotypes might be eliminated by adaption of individuals to environmental stress [33]. However, it is unclear at this point whether the gene polymorphisms that were associated with post-thaw semen quality present a direct functional effect or if they are in linkage disequilibrium with other functional SNPs. Previous studies have reported that the effect on genotype or SNP variant might be influenced by the other SNPs, suggesting the interactions of multiple SNPs on the semen quality traits [32,33]. Accordingly, polymorphisms in boar spermatozoa could be used as markers associated with causative mutations within the gene [12,26].

In this study, the variant calling procedure detects polymorphisms associated with fertilization-related traits based on their relevance in sperm motility, metabolism, reproduction, and embryo development. We described here the associations of sperm-related genetic polymorphisms with post-thaw quality of boar semen. Candidate genes with significant SNP associations, including *APPL1, PLBD1, FBXO16, EML5, RAB3C, OXSR1, PRICKLE1,* and *MAP3K20* genes, are promising markers for post-thaw semen quality, and that they might be relevant for future improvement in the selection procedure of boars for cryopreservation. We suggest that polymorphisms in genes expressed in spermatozoa could be considered as factors associated with post-thaw semen quality. However, further well-designed studies, with a larger animal population, are required to investigate the effect of gene polymorphisms in boar spermatozoa on semen freezability.

4. Materials and Methods

4.1. Animals and Semen Collections

A total of forty PLW boars were used in this study. The six boars used for RNA-Seq were stationed at the Cryopreservation laboratory, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn [8]. For the association studies, most of the ejaculates were collected from boars, which were stationed at the Cryopreservation laboratory, and a total of 51 ejaculates were collected from boars stationed at three AI centers [5]. A total of approximately 296 ejaculates, at least three ejaculates per boar, were collected from the 40 boars (during the autumn–winter period), using the gloved-hand technique. The animals were fed with a commercial porcine ration and were kept in individual pens throughout the experimental period. Water was available ad libitum. Only sperm samples with more than 70% motility and 85% normal morphology were used for cryopreservation. Animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee, Olsztyn (Poland). Experiments on boars (semen collection procedures) do not require the approval of the Local Ethics Committee (Olsztyn) from 15/01/2015.

4.2. Cryopreservation Procedure

Semen was frozen according to a cryopreservation protocol using lyophilized lipoprotein fractions of ostrich egg yolk (LPFo), as described in previous studies [39,50,55]. The LPFo-extended semen was cooled to 5 °C for 2h and diluted (2:1) with another freezing extender (89.5 mL lactose-LPFo extender, 9 mL glycerol, and 1.5 mL Orvus Es Paste). All samples (500×10^6 spermatozoa/mL) were frozen in a programmable computer freezer (Ice Cube 1810, SY-LAB, Austria), using an appropriate cooling rate [55], prior to storage in liquid nitrogen. Frozen samples were thawed in a water bath for 60 sec at 50 °C for post-thaw sperm analysis (motility and membrane integrity). Following post-thaw, the samples (50×10^6 spermatozoa/mL) were held in a water bath for 10 min at 37 °C prior to semen quality assessment.

4.3. Semen Quality Assessment

Quality assessment was performed in fresh, pre-freeze, and post-thaw semen.

4.3.1. Sperm Motility

The percentages of motile spermatozoa were assessed by the same technician throughout the study. Briefly, semen samples (6 µL) were placed on pre-warmed slide and assessed under a light microscope at 200 × magnification (Olympus BX 40, Tokyo, Japan) equipped with an attached heated stage (37 °C). Sperm motility was evaluated randomly in at least five fields per sample [5].

4.3.2. Mitochondrial Membrane Potential (MMP)

Sperm MMP was assessed with the fluorescent lipophilic cation JC-1 and propidium iodide (PI) fluorescent dyes [5,56].

4.3.3. Plasma Membrane Integrity (PMI)

Sperm PMI was assessed with the SYBR-14 and PI fluorescent probes, using the Live/Dead Sperm Viability Kit (Molecular Probes, Eugene, OR, USA) [57].

4.3.4. Normal Apical Ridge (NAR) Acrosome Integrity

A staining protocol was used to assess the sperm normal apical ridge (NAR) acrosome integrity [34,50].

4.3.5. DNA Fragmentation

The procedure used to assess sperm DNA fragmentation has been described in previous studies [39,55]. Briefly, agarose-embedded sperm samples on microscopic slides were stained with ethidium bromide and were assessed at 400× magnification under a fluorescence microscope (Olympus BX 41, Tokyo, Japan).

4.3.6. Lipid Peroxidation (LPO)

Sperm LPO was determined spectrophotometrically by malondialdehyde (MDA) production [5]. The LPO was defined as the production of nM MDA by 1×10^8 spermatozoa following 1 h incubation at 37 °C (nM MDA/10^8 spz/h).

4.4. Genomic DNA Isolation

Genomic DNA was isolated from washed sperm cells (200×10^6 spermatozoa/mL) of 40 boars (21 boars with GSF and 19 boars with PSF) using the protocol of the Sherlock AX Purification kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instructions. Lysing solution containing proteinase K and dithiothreitol was added to sperm pellets. The mixture was incubated for

60 min at 50 °C and was subjected to a column filtration and purification procedure. Genomic DNA was treated with a precipitation enhancer isopropanol, and the DNA pellets were washed 2× in 70% ethanol and air-dried (10 min) at room temperature. The isolated DNA samples were dissolved in Tris-EDTA buffer and stored at −20 °C, until further analysis.

4.5. Variant Calling and Filtering Analysis

We used the RNA-Seq datasets that were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (Bioproject: PRJNA415904; accession number SRP121647) (2018/05/27). The datasets represent RNA-Seq from three PLW boars each of the GSF and PSF groups [8].

For the RNA-Seq, the clean reads were mapped to the *Sus scrofa* reference genome from Ensembl (genome-build *Scrofa* 11.1.91) using the two-pass mapping strategy in the spliced transcripts alignment to a reference (STAR) software [58]. The BAM alignment files were processed, and single nucleotide variants (SNVs) were called by the Picard tool (http://broadinstitute.github.io/picard) and GATK [59]. The data were filtered based on read depth greater than 10 (≥10) and quality score greater than 90 (≥ 90), as described in a previous study [23]. Comparison of frequency variations of SNVs was performed using the rMATS-DVR software [60]. Each potentially heterozygous genomic position was statistically examined using the False Discovery Rate (FDR< 0.05) to identify variations in the alternate allele frequencies between boars of the freezability groups. The parameters used to identify each SNV in the freezability groups were as follows: gene position, position of *Sus scrofa* chromosome (SSC), accession number in dbSNP database (version 11.91), differences in genetic coverage of both reference and alternate allele counts, allele fraction differences, number of counts for the reference and allele fractions, and the FDR value. Validation of SNPs was performed by matching putative polymorphisms to known pig dbSNP entries using standalone BLAT v.36 [61]. The SNPs that were not present in the dbSNP were considered as unknown (novel predicted SNP). As an additional variant filtering procedure, we retained SNPs that were on the coding sequences (CDS), 3′-untranslated regions (3′-UTRs), 5′untranslated regions (5′-UTRs), putative promotor regions (approximately 200 bp from the beginning of the transcript), and non-coding sequences (ncRNAs). After multi-filtering analysis, the identified variants were annotated by the SnpEff software v.4.1 [62] and Variant Effect Predictor (VEP) Ensembl [63] to retrieve the significant SNPs (Supplementary Table S1). We searched the literature to identify the functions of the CGs in the reproductive processes, sperm physiology or QTLs, according to the Animal Genome pigQTLdb. The CGs containing variants were selected according to their polymorphism coverage in the dataset (Supplementary Table S1) or their role in reproductive processes, sperm functions, or reproduction traits based on QTLs. Thus, by considering these criteria, forty CGs with polymorphisms (Supplementary Table S4) were selected for validation using the KASP genotyping assay (LGC Genomics Ltd., Trident Industrial Estate, Hoddesdon, Hertfordshire, UK).

4.6. KEGG Pathways and GO Enrichment Analysis

The Kobas functional annotation tool (v3.0) was used to identify in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways statistical enrichment ($p < 0.05$) of the CG genes with polymorphisms [64]. Blast2Go Pro software, v.5.2.5 [65] was used to perform functional annotations, according to gene ontology (GO) categories (biological process, molecular function, and cellular components). *Sus scrofa* Ensembl database was downloaded from Ensembl BioMart Martview application to perform blastx, Blast2GO mapping, and the GO enrichment analysis. The GO significance levels ($p < 0.05$) were computed for multiple testing in the Blast2GO software program [65].

4.7. SNP KASP Genotyping Assay

The accuracy of the genotype calls was validated using the KASP genotyping assay (LGC Genomics Ltd., Trident Industrial Estate, Hoddesdon, Hertfordshire, UK). Forty SNPs were genotyped using 50 up and downstream flanking regions (Supplementary Table S5). Genomic DNA isolated from spermatozoa

of forty PLW boars was shipped to the LGC Genomics Lab (http://www.lgcgroup.com) to perform the SNP genotyping assay based on KBioscience's Kompetitive allele-specific PCR amplification. Primers designed for KASP™ genotyping procedure were performed by the LGC Genomics laboratory using the KBioscience PrimerPicker software [22]. The data were analyzed with the Kluster-caller software and SNPViewer (LGC Genomics, Ltd.; http://results.lgcgenomics.com/software/snpviewer,) to identify SNP genotypes.

4.8. Statistical Analysis

Data were statistically analyzed with the IBM SPSS Statistics 25 software package (IBM SPSS Statistics for Windows, version 25.0, IBM Corp., Armonk, NY, USA) and Statistica software package, version 12.5 (StatSoft Incorporation, Tulsa, OK, USA). The normality of the data distribution was analyzed with ANOVA assumption (Shapiro Wilk test), and the Levene's test was used to check for homogeneity of variance. The general linear modeling (GLM) procedure was used for ANOVA analysis. The effect of boar on fresh, pre-freeze, and post-thaw semen quality was analyzed using Model (1).

$Y_{ij} = \mu + B_i + e_{ij}$ (Model 1)

where Y is the measured semen quality traits; μ is the overall mean; B_i is the fixed effect of boar; e_{ij} is the random residual effect.

The effect of SNP (G) on post-thaw semen quality was analyzed using Model (2).

$Y_{ij} = \mu + G_i + e_{ij}$ (Model 2)

where Y is the measured semen quality traits; μ is the overall mean; G_i is the fixed effect of the genotype; e_{ij} is the random residual effect.

In the boar population not all possible genotypes were detected for *SCLT1*, *MAP3K20*, *MS4A2*, and *ROBO1*. The association analysis of these gene polymorphisms with post-thaw semen quality was performed using Model (3).

$Y_{ijk} = \mu + F_i + G_j + e_{ijk}$ (Model 3)

where Y is the measured semen quality traits; μ is the overall mean; F_i is the fixed effect of freezability; G_j is the fixed effect of the genotype; e_{ijk} is the random residual effect.

Pairwise comparisons were analyzed with an independent two-tailed T-test, while multiple comparisons were performed with Tukey's honest significant difference (HSD) test as appropriate. Descriptive variables are presented as the mean ± SEM. Significant differences were considered at $p < 0.05$.

The GENEPOP v.4.7.2 software package [66] (https://kimura.univ-montp2.fr/~{}rousset/Genepop.htm) was used to a) calculate the allele and genotype frequencies (Fisher's exact test; b) determine Hardy–Weinberg equilibrium (HWE) for all loci using both the Fisher's exact test and chi-square test (χ2-test), and c) examine the population differences between the two freezability groups.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/5/1902/s1. Supplementary Table S1. Identification of single-nucleotide variants (SNVs) in spermatozoa boars with good and poor semen freezability (GSF and PSF, respectively). Supplementary Table S2. KEGG pathway of enriched candidate genes (CGs) of boar spermatozoa. Supplementary Table S3. Gene Ontology (GO) of enriched terms associated with selected candidate genes (CGs) of boar spermatozoa. Table S4. Reproductive processes, sperm functions and quantitative trait loci (QTLs) for pig reproductive traits of selected candidate genes (CGs) with single nucleotide polymorphisms (SNPs). Supplementary Table S5. Single nucleotide polymorphism (SNP) markers and primer sequences used for KASP genotyping assay. References [67–92] are cited in the supplementary materials.

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Paper II

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Promoter polymorphisms in *STK35* and *IFT27* genes and their associations with boar sperm freezability

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ABSTRACT

We have shown that *STK35* and *IFT27* genes are differentially expressed in spermatozoa from boars with good and poor semen freezability (GSF and PSF, respectively). *STK35* is a stress-related gene that is implicated in spermatogenesis, whereas *IFT27* is a motility-related gene that is mainly involved in intracellular protein transport. In this study we hypothesized that polymorphic variants in the 5′-flanking regulatory regions of *STK35* and *IFT27* genes could contribute to differences in semen freezability. We also predicted the interactions of the polymorphic variants with transcription factors on the gene promoter activity, using bioinformatics. The 5′-flanking region sequences of the *STK35* and *IFT27* were PCR amplified and analyzed by Sanger sequencing method. Protein expression in *STK35* and *IFT27* was determined in pre-freeze (PF) and frozen-thawed (FT) spermatozoa, using western blotting analysis. Sanger sequencing revealed a single nucleotide polymorphism (SNP) rs327863835 (C > T) in *STK35* promoter, while two SNPs (rs337563873, A > T; rs331520020, T > C) were detected in *IFT27* promoter. *STK35* and *IFT27* promoter polymorphisms showed significant allele frequency differences between the GSF and PSF groups. Using bioinformatics approaches, we predicted that SNPs resulted in the generation of additional transcription factor binding sites for NFATC2, ELK1 and GR-β, which appeared to enhance or repress the promoter activity of *STK35* or *IFT27* in either freezability group. Wide variations in STK35 and IFT27 protein expression were observed among the boars, however, significantly higher protein expression was detected in *IFT27* in FT spermatozoa of the GSF group. We suggest that the upstream variants, detected in *STK35* and *IFT27* promoters, might regulate the transcriptional activity of the genes by affecting their potential binding of transcription factors. The results indicate that the allelic variants in *STK35* and *IFT27* could be considered as potential genetic markers for predicting boar sperm freezability.
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1. Introduction

The freezability of boar semen is affected by several factors, such as ejaculate fractions, seminal plasma proteins and individual variations [1–4]. More recently, studies have confirmed that genes expressed in boar spermatozoa are associated with semen freezability [5–7]. There is substantial evidence indicating that polymorphisms in the 5′-flanking region of the genes expressed in spermatozoa could affect transcriptional activity, and are associated with semen quality [8–10]. Moreover, differentially expression of genes are influenced by the presence of transcription factors (TFs) that may act to activate or repress gene expression depending on the presence of other regulating features [11–14].

We have considered that *STK35*, a stress-related gene, and *IFT27*, a motility-related gene, would be of particular interest with regards to semen freezability. In our recent study we have shown that the expression of serine/threonine kinase 35 (*STK35*) and intraflagellar transport 27 (*IFT27*) genes, detected by a Bioconductor package (DESeq), was up-regulated in the poor freezability ejaculates [7]. *STK35* is expressed in the spermatogenic cells and is reported to contribute to cellular stress response in the rodent testis [15,16].

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Evidence has shown that *STK35* gene is highly expressed in the testis, and it has been suggested that the nuclear-localized importin-α2 (IMP-α2) protein is one of the mechanisms that could regulate *STK35* transcription during spermatogenesis [15,17,18]. The expression of *STK35* was up-regulated in early embryo development and was related to protein phosphorylation [19].

It is noteworthy that the IFT-related proteins are expressed during spermatogenesis, and have distinctive role in the progress of the sperm development [20,21]. Among the IFT genes, *IFT27 (RABL4)*, which is highly expressed in the testis is of particular interest because of its critical role in sperm motility and metabolism [20–22]. Moreover, IFT27 is a component of *IFT-B*, and disruption of *IFT-B* genes usually results in flagellar abnormality [22]. Evidence has shown that IFT27, a core component of the IFT complex machinery, is localized in the sperm midpiece region and through its interactions with *RABL2* (a member of RAS oncogene facility-like 2) is involved in transporting proteins required for glycolysis to the sperm fibrous sheath [[20,23]). It has been demonstrated that IFT25/IFT27 complex plays an important role in assembling the core axoneme structure of spermatozoa [21]. Moreover, the IFT27 protein is essential for sperm flagella assembly, spermatogenesis and male fertility in the mice [21,22]. The role of the IFT-related proteins in sperm function has not been elucidated as yet.

More recently, we have confirmed that single nucleotide polymorphisms (SNPs) in the promoters of genes expressed in boar spermatozoa affect the gene transcriptional activity, and are associated with poor freezability ejaculates [10]. We have reported that the up-regulation of *STK35* and *IFT27* was more marked in poor freezability ejaculates [7], and in this study we hypothesized that SNPs in the gene promoters could be associated with semen freezability. Therefore, the objectives of this study were to i) identify polymorphisms in the 5′-flanking regions in *STK35* and *IFT27* genes expressed in spermatozoa, ii) predict the effect of polymorphic variants in *STK35* and *IFT27* promoters on transcription factor (TF) binding ability, using bioinformatics, and iii) analyze the protein expression of STK35 and IFT27 in pre-freeze (PF) and frozen-thawed (FT) spermatozoa, using Western blotting analysis.

2. Materials and methods

2.1. Chemicals and media

Chemicals were bought from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated. Fluorescent probes, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbo-cyanineiodide (JC-1) and propidium iodide (PI), were bought from Molecular Probes (Eugene, OR, USA), while thiobarbituric acid (TBA) was purchased from Merck KGaA (Darmstadt, Germany).

2.2. Animals and semen collections

This is a follow-up study in which the sperm-rich fractions (SRFs) were collected from eleven Polish large white (PLW) boars (average aged 2 yr) [10]. A total of 33 and 36 SRFs were collected from boars classified as having good and poor semen freezability (GSF and PSF, respectively), as indicated in our previous study [10]. All animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee.

2.3. Experimental design

Sperm motility is one of the most important factors affected by the freezing-thawing process and is most widely used to assess post-thaw semen quality because it correlates with fertility outcome [24,25]. Total motility (TMOT) of FT spermatozoa is one of the main traits used to classify the freezability of boar semen [7,10,26,27]. Post-thaw TMOT cut-off threshold value of 30% (GSF >30% and PSF <30%) was used for the classification of the freezability groups [7,10,26–28]. According to this classification, five boars were allocated to the GSF group (G01, G04, G09, G16 and G17), while six boars were assigned to the PSF group (P30, P36, P37, P38, P39 and P40). The classification of the semen freezability was highly consistent within boars following post-thaw sperm assessment. Parametric variables were analyzed with the GLM procedure, whereas non-parametric variables were analyzed with the Kruskal-Wallis ANOVA test.

2.4. Semen processing procedure

The SRF samples were processed according to a cryopreservation protocol [3,29,30]. Briefly, the SRFs were diluted (1:4) in Beltsville Thawing Solution (BTS) and cooled at 17 °C, before being centrifuged (800×*g*, 10 min). The recovered sperm pellets were re-suspended in an extender containing 11% lactose (w/v) and lipoprotein fractions of ostrich egg yolk (LPFo) extender, and cooled for 2 h at 5 °C. Following cooling, the semen samples were diluted (2:1) in another freezing extender (89.5 mL lactose-LPFo extender, 9 mL glycerol and 1.5 mL Orvus Es Paste). Semen samples (500×10^6 spermatozoa/mL) were packaged into 10-mL sterilized aluminum tubes (Factory of Medical Materials, Polfa, S.A. Boleslawiec, Poland) and loaded onto a programmable computer freezing machine (Ice Cube 1810, SY-LAB, Austria), using an appropriate cooling rate [30]. Frozen semen samples were stored in liquid nitrogen prior to post-thaw analysis. Samples were thawed in a water bath for 60 s at 50 °C and were diluted in BTS (50 × 106 spermatozoa/mL) before post-thaw sperm analysis.

2.5. Semen quality assessment

The SRFs were diluted (1:1) in BTS for the assessment of the PF sperm characteristics prior to cooling at 17 °C.

2.5.1. Motility and motion characteristics

Sperm motility and motion characteristics were assessed with the computer-assisted semen analysis (CASA) system (HTR-IVOS 12.3, Hamilton Thorne Biosciences, MA, USA). Briefly, aliquots of sperm samples (5 μL) were placed on a Makler chamber (Counting Chamber Makler, Sefi-Medical Instruments Ltd, Israel) pre-warmed at 38 °C on a thermal plate and a minimum of 200 spermatozoa from five random fields were analyzed using a 10 × objective (negative phase contrast) [7]. The software settings used for the CASA system were those recommended by the manufacturer for analysis of boar spermatozoa, and were extensively described in a previous study [7]. The sperm parameters assessed by the CASA system included TMOT (%), progressive motility (PMOT; %), rapid movement (%), velocity straight line (VSL; μm/s), velocity average-path (VAP; μm/s), velocity curvilinear (VCL; μm/s), amplitude of lateral head displacement (ALH; μm) and beat cross frequency (BCF; Hz).

2.5.2. Assessment of sperm membrane integrity

Two fluorescent probes, JC-1 and PI, were used to assess sperm mitochondrial membrane potential (MMP) [31,32]. Briefly, sperm samples (30×10^6 spermatozoa/mL) were washed by centrifugation (600×*g*, 5 min) and the pellets were re-suspended in a HEPES saline medium. Aliquots of washed sperm samples were incubated in JC-1 (1 mg JC-1/mL in anhydrous dimethyl sulfoxide) and PI solutions (2.4 μM PI in Tyrode's salt solution) for 15 min at 37 °C, and were examined at 600× magnification under a fluorescence microscope (Olympus CH 30, Olympus Optical Co. Ltd. Tokyo,

Japan), equipped with dichroic mirrors for blue (DMB) and green excitations (DMG) for JC-1 and PI, respectively. Spermatozoa that exhibited orange-red fluorescence in the midpiece region were considered as viable sperm cells with high MMP, whereas non-viable spermatozoa with low MMP fluoresced red in the head and green in the mid-piece region. A minimum of 100 sperm cells were examined per slide. Each slide was analyzed in duplicate.

The percentage of spermatozoa with plasma membrane integrity (PMI) was monitored by dual fluorescent staining with SYBR-14 and PI (Live/Dead Sperm Viability Kit; Molecular Probes, Eugene, OR, USA) [33]. Briefly, sperm samples (30×10^6 spermatozoa/mL) were incubated in SYBR-14 (1 mM SYBR-14 solution in HEPES-BSA, pH 7.4) and PI solutions (2.4 mM PI in Tyrode's salt solution) for 10 min at 37 °C, and were examined at 600× magnification under a fluorescence microscope (Olympus CH 30). A minimum of 100 sperm cells were examined per slide, duplicate slides being prepared for each sample.

The percentage of spermatozoa with normal apical ridge (NAR) acrosome integrity was assessed, using a modified Giemsa staining protocol [3,34]. Sperm samples were spread on slides, air-dried, and fixed by immersion in formal-saline solution, before being stained with the Giemsa staining solution. At least 100 spermatozoa were assessed at 1000× magnification under a bright light microscope equipped with oil immersion lens (Olympus B×41, Olympus, Tokyo, Japan), and were considered as sperm cells with intact apical or damaged apical ridge. Each slide was analyzed in duplicate.

2.5.3. DNA fragmentation

The comet assay was used to assess sperm DNA fragmentation [29,30]. Briefly, sperm samples were washed by centrifugation (800×*g*, 5 min) and the pellets (10×10^6 spermatozoa/mL) were re-suspended in phosphate buffered solution. Prior to treatment with a lysing buffer. Sperm cells embedded in agarose on microscopic slides were stained with ethidium bromide, and a minimum of 200 cells per slide were examined in random fields at 400× magnification under a fluorescence microscope (Olympus B×41, Tokyo, Japan). Spermatozoa were classified as fragmented DNA on non-fragmented DNA sperm cells. All slides were analyzed in duplicate.

2.5.4. Lipid peroxidation (LPO)

The TBA assay used to assess the LPO potential [32,35]. Briefly, sperm samples (100×10^6 spermatozoa/mL) were washed and the pellets were re-suspended in a Tris buffer (pH 8.0) containing 0.01 M ferrous sulfate and 0.1 M sodium ascorbate, prior to incubation for 60 min at 37 °C. Trichloracetic acid (TCA) was added to the reaction mixture before being washed (7800×*g*, 15 min) and used for the TBA reaction [35]. The absorbance of the solution was measured, using a spectrophotometer (Beckman DU-62, Beckman Instruments Inc., Fullerton, CA, USA) and LPO was defined as nanomoles of malondialdehyde (MDA) formed by 10^8 spermatozoa after 1 h incubation at 37 °C (nM MDA/10^8 spermatozoa).

2.6. Genomic DNA extraction

We extracted DNA from the ejaculated spermatozoa with the Sherlock AX Purification kit (A&A Biotechnology, Poland) as previously described in study [27]. The Nanodrop Spectrophotometer (ND-1000, NanoDrop, Thermo Fisher Scientific Inc.) was used to analyze DNA yields and quality. Purified genomic DNA samples were suspended in a Tris-EDTA buffer and stored at −20 °C, until required.

2.7. Primer design, PCR amplification and sanger sequencing

Primers for *STK35* and *IFT27* were designed as described in our previous study [10]. Briefly, the Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design the PCR primers from *STK35* and *IFT27*. The 5′-flanking sequences of *STK35* and *IFT27* were obtained from the genome assembly *Sscrofa* 11.1 (GCA-000003025.6; Ensembl database), using the position of 5′-terminal nucleotide (nt) of each transcript (transcription start site, +1 nt) as the border line between the regulatory and structural parts of each gene. We used the DNA fragments containing about 800 bp 5′-directed from transcription start site and about 200 bp 3′-directed from the transcription start point were used as the template for the primer design (Table 1).

The PCR reactions were performed with the KAPA Taq PCR kit (KAPA Biosystems, Boston, USA) according to the manufacturer's instructions, using Biometra T3 thermocycler (Analytic Jena Company, Germany). All amplicons were produced employing the PCR touch-down thermal procedure [36]. Prior to sequencing, the PCR products specificity and quantity were validated by 1.5% agarose gel electrophoresis with ethidium bromide [10]. The PCR amplification products were analyzed by bidirectional Sanger sequencing (Genomed S.A. Company, Warsaw, Poland). We identified the DNA polymorphisms by Clustal W multiple alignment analysis [37], using the BioEdit Sequence Alignment Editor ver. 7.2.6 [38]. Visual examinations of DNA sequences of the ABI chromatogram files (Chromas ver. 2.6.6.; www.technelysium.com.au) were performed to correct the base-calling and sequencing errors.

2.8. In silico analysis of binding sites of transcription factors (TFs)

In silico analysis of the binding sites of TFs was performed to assess the potential biological functions of SNPs in *STK35* and *IFT27* promoters, using the ALGGEN via PROMO analysis tool (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) [39,40]. The TF binding sites, recognized in the TRANSFAC (TRANScription FACtor) database ver. 8.3), were used to construct specific binding site weight matrices for the TF binding site predictions [41]. The similarity of a sequence variant to a matrix was determined [42], and the maximum matrix dissimilarity rate was set at 5%. The TFs, detected in this study, and their biological functions are shown in Table 2.

2.9. Western blotting analysis

Proteins were extracted from the PF and FT spermatozoa of each boar, according to a previously described method [3]. Three PF and FT sperm samples were randomly selected from each boar for the analysis The supernatant obtained from washed sperm pellets was stored at −80 °C, until required. Following protein measurements [43], western blotting analysis was performed in sperm extracts from 5 boars of the GSF group (G01, G04, G09, G16 and G17) and 6 boars of the PSF group (P30, P36, P37, P38, P39 and P40) to identify protein expression in STK35 and IFT27. Protein samples (30 μg/lane) were separated by SDS-PAGE [44] and transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) for immunodetection. We performed electroblotting according to previously described method [45].

The specific characteristics of the immunoblot antibodies used for western blotting analysis are shown in Table 3. Following blocking non-specific binding sites with 5% nonfat milk in Tris-buffered saline, containing 0.05% v/v Tween 20, TBST (MP Biomedicals LLC, Santa-Ana, CA, USA), blots were incubated with rabbit polyclonal STK35 and IFT27 (RABL4) antibodies overnight at 4 °C. Rabbit beta-actin antibody (ACTB, A2066:1:500; Sigma

Table 1
Primers used in polymerase chain reaction (PCR) and sequencing analysis.

Ensembl	Gene name	Gene symbol	Primer sequence	Amplicon size (bp)
ENSSSCG00000007179	Serine/threonine kinase 35	*STK35*	F: 5′-GCCCAATCACAGGCTAGAAC-3′ R: 5′-TAATGGTGTTCCCTGGTGGT-3′	745
ENSSSCG00000000139	Intraflagellar transport 27	*IFT27*	F: 5′-GGCAGAGTAGATGGGAGCAG-3′ R: 5′-ACCACTTTCCTTCTCGCTCA-3′	706

Table 2
Transcription factors (TFs) and their biological functions.

Full name	TFs	TRANSFAC database with accession numbers	Description of transcription factor (TF)	References
Nuclear factor of activated T cells 2	NFATC2	T00550	This protein is present in the cytosol and only translocates to the nucleus upon T cell receptor stimulation, where it becomes a member of the nuclear factors of activated T (NFAT) cells transcription complex.	[13,47]
ETS transcription factor	ELK1	T00250	A member of the ETS family of transcription factors and of the ternary complex factors (TCFs). Different domains of the TCFs are involved in transcriptional activation or repression.	[47,50,52]
Nuclear receptor subfamily 3 group C member 1 (Glucocorticoid receptor beta)	NR3C1 (GR-β)	T01920	Receptor for glucocorticoids, with dual mode of action: as a TF that binds to the glucocorticoid response elements (GREs) and as a modulator of the activity of several TFs. NR3C1 is involved in activation or repression of gene transcription.	[54,55]
CCAAT/enhancer binding protein alpha	C/EBPα	T00105	C/EBP is a DNA-binding protein that recognizes two different motifs: the CCAAT homology common to many promoters and the enhanced core homology common to many enhancers.	[47]

Chemical Company) was used as the loading control. Following washing, the membranes were incubated for 2 h at room temperature with anti-rabbit IgG, horseradish peroxidase (HRP)-linked secondary antibody (111-035-003; 1:1000; Jackson ImmunoResearch, Baltimore Pike, PA, USA). Protein detection was performed by enhanced chemiluminescence (ServaLight CL EOS Substrate kit (Serva, Heidelberg, Germany). All images were captured with the G:BOX iChemi XR imaging system (SynGene, Cambridge, UK), and protein bands were quantified using MultiAnalyst 1.1 software. The molecular weights of the protein were determined using the Molecular weight standard (Pre-stained Protein Ladder™, Unstained Protein Standards, Bio-Rad.). Values are expressed as the total signal intensity inside the boundary of a band measured in pixel intensity units/mm^2 – optical density (OD), using beta-actin, ACTB (Sigma Aldrich) as the protein control to normalize the volume of protein expression [7,46].

2.10. Statistical analysis

Statistical analysis was performed with the Statistica software package, version 13.0 (TIBCO Software Inc., CA, USA; StatSoft Polska, Kraków, Poland). For variables that were normally distributed, the following linear model was used to analyze the effect of boar on the PF or FT sperm quality traits [10,27]:

$$Yij = \mu + \beta i + eij,$$

where Y is the measured sperm quality traits; μ is the overall mean of each trait; βi is the fixed effect of boar; eij is the random residual effect.

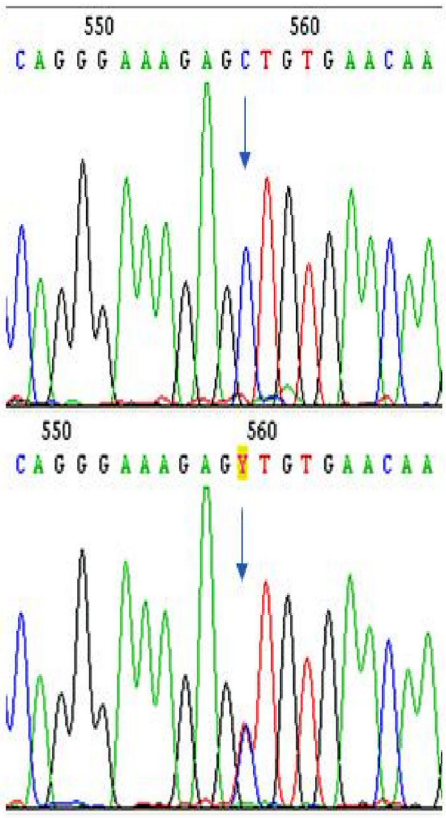
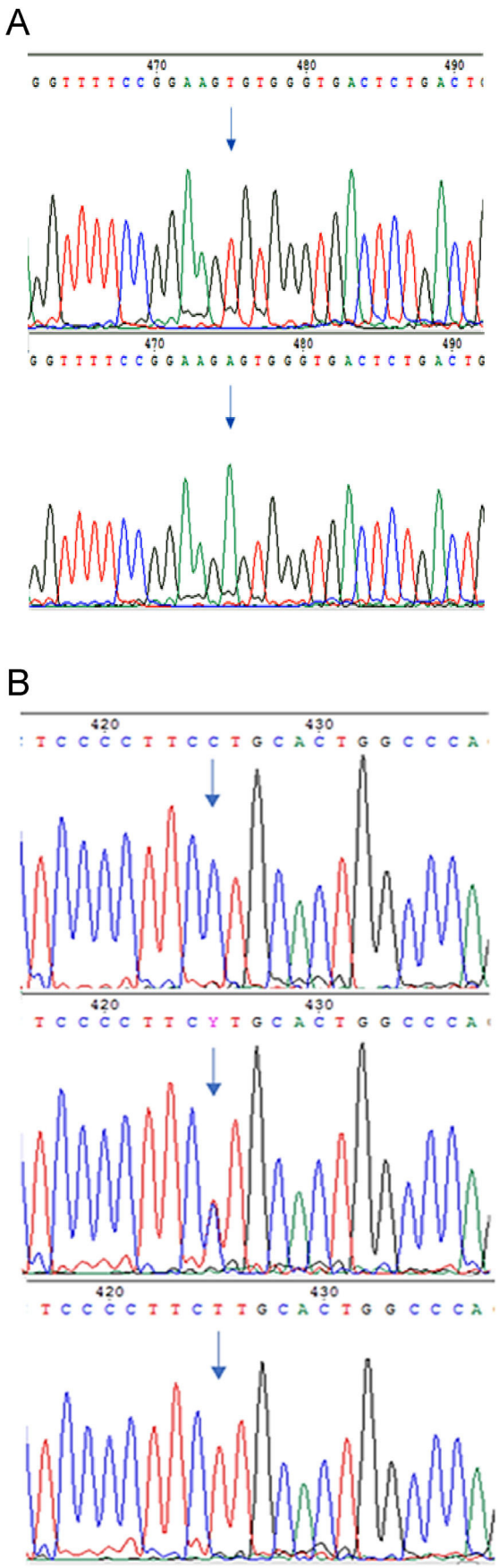


Fig. 1. Direct sequencing of a 745-bp 5′-flanking region of *STK35* gene showing a single nucleotide polymorphism (rs327863835) represented by two genotypes, C/C (upper panel) and C/T (lower panel).

Table 3
Specific immunoblot antibodies used in western blotting analysis.

Target protein	Gene symbol	UniProtKB accession number	Molecular weight	Dilution	Host	Conjugate	Company	Catalog number
Serine/threonine kinase 35	*STK35*	F1S890	58 kDa	1:500	rabbit	polyclonal	Thermo Fisher Scientific	PA5-14082
Intraflagellar transport 27	*IFT27 (RABL4)*	F1SKJ7	21 kDa	1:500	rabbit	polyclonal	Thermo Fisher Scientific	PA5-43155

Thermo Fisher Scientific (Waltham, MA, USA).



Five boars were allocated to the GSF group, while six boars were assigned to the PSF group, according to post-thaw TMOT analysis. The independent two-tailed T-test was used to assess differences between the GSF and PSF groups. Variables which data were not normally distributed (VSL, VAP, VCL, ALH and BCF) were analyzed with the Kruskal-Wallis ANOVA test, and significant differences between the GSF and PSF groups were assessed with the Mann-Whitney *U* test.

The Pearson's chi-square test (χ^2) was used to determine the differences in the allele frequencies between the GSF and PSF groups [10]. The Kruskal-Wallis ANOVA with a multiple comparison test was used to analyze the protein expression levels in the PF and FT spermatozoa among the groups: PF-GSF, PF-PSF, post-thaw GSF and post-thaw PSF groups. Descriptive variables are presented as the mean ± SEM. Values were considered significant at $P < 0.05$.

3. Results

3.1. Semen quality assessment

There were no significant differences ($P > 0.05$) in the PF sperm characteristics among the boars (Supplementary Table S1 and Supplementary Table S2). Post-thaw analysis showed that most of the CASA-analyzed sperm parameters were significantly higher ($P < 0.05$) in the GSF group than in the PSF group (Supplementary Table S3). No significant differences ($P > 0.05$) were observed with respect to post-thaw ALH or BCF values (Supplementary Table S3). It was found that post-thaw sperm membrane integrity, represented by MMP, PI and NAR acrosome integrity, were significantly higher ($P < 0.05$) in the GSF group than in the PSF group (Supplementary Table S3). Sperm DNA damage and LPO were markedly higher ($P < 0.05$) in the PSF group following freezing-thawing (Supplementary Table S3).

3.2. Polymorphism analysis

In this study we performed direct sequencing of a 745-bp and 706-bp of the 5′-flanking regions (upstream) of the *STK35* and *IFT27* genes (Table 1), and bioinformatics was performed to predict the significant importance of the interactions of the polymorphic variants with TFs on the transcriptional activity in the genes.

Sanger sequencing revealed a single nucleotide polymorphism (SNP) rs327863835 (C > T) in *STK35* promoter (Fig. 1), while two SNPs (rs337563873, A > T; rs331520020, T > C) were detected in *IFT27* promoter (Fig. 2). The representative electropherograms of two genotypes (C/C and C/T, indicated by arrows) of *STK35* promoter polymorphism (rs327863835) are shown in Fig. 1. Markedly higher ($P < 0.05$) frequency of the C allele in *STK35* rs327863835 (C > T) was observed in the GSF group, while a higher ($P < 0.05$) frequency of the T allele was more marked in the PSF group (Fig. 1 and Table 4). The representative electropherograms of two genotypes (T/T and A/T, represented by arrows) of *IFT27* promoter polymorphisms (rs337563873) are shown in Fig. 2A. In addition, three genotypes (C/C, T/C and T/T, represented by arrows) were detected in the *IFT27* promoter polymorphism (rs331520020), as shown in Fig. 2B. Significantly higher ($P < 0.05$) frequencies of the A and T alleles were observed in *IFT27* rs337563873 (A > T) of the PSF and GSF groups, respectively (Fig. 2A; Table 4). Similarly, boars of the PSF and GSF groups exhibited significantly higher ($P < 0.05$) frequencies of the T and C alleles in *IFT27* rs331520020 (T > C), respectively (Fig. 2B; Table 4).

Fig. 2. Direct sequencing of a 706-bp 5′-flanking region of *IFT27* gene showing two single nucleotide polymorphisms: (A) rs337563873 is represented by two genotypes, T/T (upper panel) and A/T (lower panel) and (B) rs331520020 is represented by three genotypes, C/C (upper panel), T/C (middle panel) and T/T (lower panel).

Table 4
Allele frequencies of chi-square test of single nucleotide polymorphisms (SNPs) in the 5′-flanking regions and biological functions of *STK35* and *IFT27* genes expressed in boar spermatozoa.

Locus	SNP ID	Allele1/Allele 2	Grup	Allele1 frequency	Allele 2 frequency	χ^2 test	*P*-level	Biological functions	References
STK35	rs327863835	C/T	GSF PSF	0.30 0.00	0.70 1.00	4.16	$P < 0.05$	spermatogenesis protein phosphorylation caspase-independent cell death response to oxidative stress	[15,16]
IFT27	rs337563873	A/T	GSF PSF	0.40 1.00	0.60 0.00	9.90	$P < 0.01$	flagellum assembly spermatid elongation sperm motility	[20–23]
	rs331520020	T/C	GSF PSF	0.30 0.83	0.70 0.17	6.41	$P < 0.05$	intracellular protein transport sperm metabolism signal transduction	

GSF – good semen freezability; PSF – poor semen freezability.

3.3. In silico analysis of TF binding sites

It was predicted that polymorphisms in the 5′-flanking sequences of *STK35* and *IFT27* promoters resulted in the generation (gain; +) of a new TF binding site or the removal (loss; −) of the original TF, as shown in Table 5. Predictive analysis showed that *STK35* rs327863835 resulted in the addition of a new TF binding site for NFATC2 (Table 5). Furthermore, *IFT27* rs331520020 resulted in the addition of a new binding site each for ELK1 and NR3C1 (GR-β), and the removal of the natural binding site for C/EBPα (Table 5). *IFT27* promoter contained ELK1 and GR-β motifs with low dissimilarity scores, indicating a high probability of binding (Table 5).

3.4. Western blotting analysis

Western blotting analysis showed varying expression of STK35 protein in the PF and FT spermatozoa harvested from boars of the GSF and PSF groups (Fig. 3A). Significant variations in the expression of STK35 protein (Kruskal-Wallis ANOVA test, $df = 3$, $P < 0.005$) were observed among the groups. Significantly lower ($P < 0.05$) relative expression of STK35 protein was observed in FT spermatozoa compared with the PF spermatozoa (Fig. 3B). No marked differences ($P > 0.05$) were observed between the freezability groups of either the PF or FT spermatozoa (Fig. 3B). Significant variations in the expression of IFT27 protein (Kruskal-Wallis ANOVA test, $df = 3$, $P < 0.007$) were observed among the groups. Blots showing IFT27 protein expression in the PF and FT spermatozoa are indicated in Fig. 4A. The relative protein expression of IFT27 did not differ ($P > 0.05$) between the freezability groups of the PF spermatozoa (Fig. 4B). It was found that FT spermatozoa of the GSF group exhibited significantly higher ($P < 0.05$) expression of IFT27 protein compared with the other groups (Fig. 4B).

4. Discussion

In a previous study we have shown that *STK35* and *IFT27* were up-regulated in spermatozoa from boars of the PSF group [7], and we hypothesized that promoter polymorphisms in these genes could be one of the mechanisms responsible for the differences in gene expression between the freezability groups. Using Sanger sequencing, we validated the presence of promoter polymorphisms in several genes expressed in boar spermatozoa and confirmed that the polymorphic variants were mainly associated with boars of the PSF group [10]. Likewise, we have found that polymorphism in *STK35* promoter region was more marked in boars of the PSF group. By contrary, a higher frequency of polymorphism in *IFT* promoter was detected in boars of the GSF group. Among the three polymorphisms detected in the gene promoters, predictive analysis showed that *IFT27* rs337563873 (A > T) did not affect the TF binding site.

The results of the present study show that *STK35* rs327863835 (C > T) resulted in the addition of a new TF binding site for NFATC2. As confirmed in previous studies, polymorphism in the promoter region of a gene changed the DNA sequences of the TF motif, thereby affecting the process of the gene regulation [14,47]. In this study, we suggest that the higher frequency of the T allele in rs327863835 (C > T), observed in the PSF group, could predispose *STK35* to altered expression. Furthermore, C > T polymorphism was predicted to generate the addition of a new TF binding site for NFATC2 on *STK35* promoter. It is noteworthy that the NFAT family of TFs encodes five distinct proteins, in which four NFAT family members (NFATC1-4) are dephosphorylated by calcineurin, a Ca^{2+}-dependent serine/threonine phosphatase, and translocated to the nucleus, where they bind to the target DNA sequences [13,47]. Moreover, the high expression of NFATC2, detected in spermatocytes and spermatids, could play an important role in immune response and spermatogenesis [13] Furthermore, it has been demonstrated that NFATs influenced mostly target genes that were up-regulated in undifferentiated spermatogonia [13]. Notably, NFATs can activate the transcription of several genes, and NFATC2 is most identified as the major NFATs contributing to a biological response [48]. Our findings strongly suggest that the addition of a new TF binding site for NFATC2 on *STK35* promoter augmented the gene transcriptional activity, resulting in its up-regulation, as

Table 5
Prediction of transcription factor (TF) binding sites associated with polymorphisms in the 5′-flanking regions of *STK35* and *IFT27* genes expressed in boar spermatozoa.

Locus	SNP ID	*Chromosome position	Allele 1/ Allele 2	TF	5′-flanking sequence recognized by TF	TF binding site gain (+) or loss (−) by polymorphism	TF consensus sequence	Dissimilarity (%)
STK3	rs327863835	17:33412895	C/T	NFATC2	GGAAAGAGT	+	GGAAAAAATT	2.51
IFT27	rs331520020	5:11000178	T/C	ELK1	CTTCCTGC	+	GAAGGACG	0.00
				NR3C1 (GR-β)	TTCCT	+	AGGAT	0.44
				C/EBPα	TTGCAC	–	TTGCTC	0.45

Polymorphic nucleotide is indicated in red; *Chromosome location assigned according to genome assembly *Sscrofa* 11.1 (GCA_000003025.6).

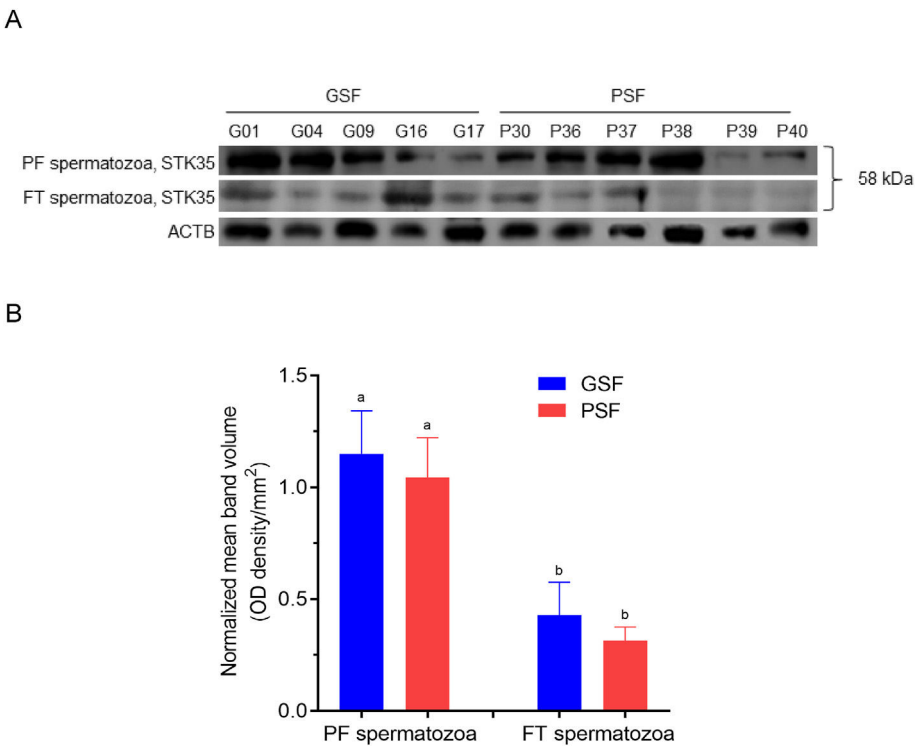


Fig. 3. Expression levels of STK35 protein in pre-freeze (PF) and frozen-thawed (FT) spermatozoa from boars classified as having good and poor semen freezability (GSF and PSF, respectively. (A) Western blotting analysis of STK35 protein expression. (B) Relative abundance of STK35 protein expression. Each column indicates the mean (±SEM) of the normalized value of protein expression from five and six boars of the GSF (G01, G04, G09, G16, and G17) and PSF (P30, P36, P37, P38, P39, and P40) groups, respectively. Beta-actin (ACTB) was used as the standard to normalize the relative expression of the analyzed protein. Values with different letters (a and b) are significant at $P < 0.05$.

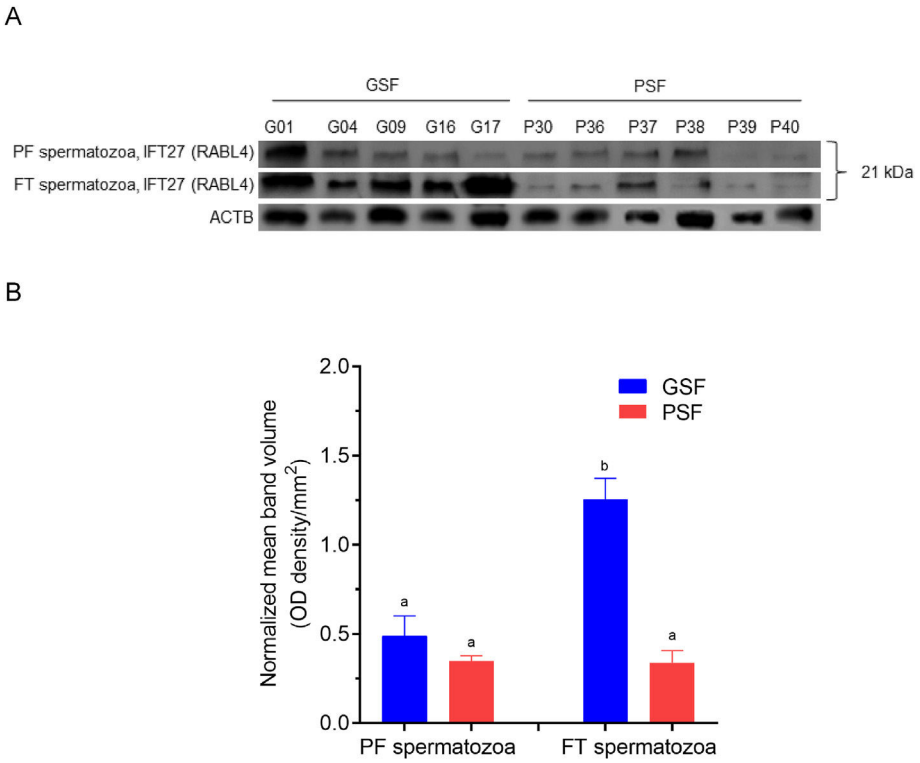


Fig. 4. Expression levels of IFT27 protein in pre-freeze (PF) and frozen-thawed (FT) spermatozoa from boars classified as having good and poor semen freezability (GSF and PSF, respectively. (A) Western blotting analysis of IFT27 protein expression. (B) Relative abundance of IFT27 protein expression. Each column indicates the mean (±SEM) of the normalized value of protein expression from five and six boars of the GSF (G01, G04, G09, G16, and G17) and PSF (P30, P36, P37, P38, P39, and P40) groups, respectively. Beta-actin (ACTB) was used as the standard to normalize the relative expression of the analyzed protein. Values with different letters (a and b) are significant at $P < 0.05$.

observed in boars of the PSF group [7]. Further evidence for another regulatory mechanism of *STK35* transcriptional activity is provided by IMP-α2, which can access the promoter region of the gene and increase its transcription in response to oxidative stress exposure [15,17,18,49]. This concurs with increased oxidative stress triggered by the up-regulation of inflammatory and apoptosis-related genes, observed in boars of the PSF group [7]. These findings are consistent with the notion that the overexpression of *STK35* is associated with increased non-apoptotic cell death in the testicular cells under oxidative stress conditions [15,16]. We suggest that the *STK35* up-regulation in spermatozoa from boars of the PSF group could affect the sperm's response to the freezing-thawing process, resulting in increased susceptibility to cryo-induced damage. However, the functional significance of *STK35* up-regulation in sperm survival remains to be explored.

Notably, *IFT27* rs331520020 (T > C) resulted in the elimination of the natural TF site for C/EBPα and addition of two new transcriptional binding sites (ELK1 and NR3C1), thereby affecting the regulation of *IFT27* expression. The effect of the removal of C/EBPα binding site on *IFT27* promoter activity is not precisely known. It is noteworthy that ELK1 is involved in the modulation of expression of proteolytic-based genes and cytoskeletal regulation genes [50,51]. Moreover, ELK1 can function as a transcriptional repressor of its target gene, which is mediated by sumoylation and dephosphorylation [52]. More importantly, the C-terminal region of ELK1 possesses a repression domain, the R motif, which can suppress the transcriptional activity of ELK1 [53]. Evidence has been shown that the ELK1 R motif is a strong repression domain that can act in *trans* and *cis* to reduce the activity of different activation domains [53]. As the C allele is more frequent in the GSF group, which is characterized by the down-regulation of *IFT27* [7], we speculate that ELK1 could function as a repressor of the gene expression in the GSF group. Consequently, the T allele, which is more abundant in the PSF group could potentially disturb ELK1 binding site, thus resulting in *ITF27* up-regulation [7]. We suggest that ELK1, with a repressive domain, seems to be involved in *IFT27* down-regulation in the GSF group. It may worth further investigations to explain the significance of *IFT27* overexpression in sperm cryo-survival.

Evidence has shown that the action of glucocorticoid (GC) action is mediated via glucocorticoid receptor, which belongs to a nuclear receptor superfamily (NR3C1) and functions as a ligand-activated TF to activate or repress the transcriptional activity of numerous glucocorticoid responsive genes [54–56]. The NR3C1 is highly expressed in the Leydig cells and is involved in steroidogenesis, and as a hormone-associated regulator, could contribute to the regulation of germ cell apoptosis [56]. Moreover, NR3C1 receptor can be differentially spliced into two major isoforms, GR-α and GR-β, in which GR-α is mainly responsible for most of GR-mediated transcriptional activity, mainly through its interaction with the GC response elements (GRE), while the GR-β does not have a ligand-binding capacity [54,57]. Although the physiological role of GR-β is still unclear, it is suggested to act as a dominant negative inhibitor of transactivation induced by GR-α [58]. Accordingly, GR-β does not bind glucocorticoids and does not directly regulate the GRE-containing glucocorticoid-responsive gene promoters [57]. Evidence has shown that the negative effect of GR-β activity on some GRE-driven gene promoters, such as fibronectin, and the overexpression of GR-β resulted in the down-regulation of several genes, suggesting that GR-β could modulate the mRNA expression of some of its responsive genes [57]. Of interest is the modulation of the transcriptional activity of response genes by GR-β through mutual protein-protein interactions with other TFs, which could contribute to the repressive activity of GR-β [57]. In a previous study such mechanism of repressive transcriptional activity of GR-β was demonstrated for activator protein-1 (AP-1) and nuclear factor κB (NF-κB) [59]. We are unclear whether such mechanism of regulation could be a plausible explanation for the down-regulation of *IFT27* in boars of the GSF group [7]. It is noteworthy that the exact role of the intrinsic transcriptional activity of GR-β in GRE-driven gene promoters remains to be elucidated. Our results confirm that the presence of GR-β binding site on the *IFT27* promoter could suppress the transcriptional activity of the gene, thereby resulting in its down-regulation, as shown in boars of the GSF group [7]. It seems that *IFT27* down-regulation in the GSF group could predispose spermatozoa to reduced cryo-damage compared with the PSF group [7].

Variation in protein abundance in mature spermatozoa can be due to several factors, namely degradation, inefficient translation and post-translational modifications [60–62]. Moreover, protein degradation and membrane damage related to cryo-injury could be responsible for reduced protein levels in FT spermatozoa [61,63] The underlying mechanisms responsible for protein abundance in the FT spermatozoa are poorly understood. Accumulating evidence has suggested that higher level of tyrosine phosphorylation is one of the mechanisms responsible for increased protein expression in FT spermatozoa than in the fresh PF spermatozoa [61,64]. According to Bogle et al. [63], increased protein expression in FT spermatozoa is most likely the consequences of a combination of several processes, including protein degradation, post-translational processing and membrane damage. More recently, it has been reported that the abundance of HSP70 protein in FT bull spermatozoa was not accompanied by improved sperm functions, but rather by capacitation-like events, probably resulting in the synthesis of the protein [65]. It is plausible that these processes could be responsible for the variations in STK35 or IFT27 protein expression levels observed between the PF and FT spermatozoa. However, the reason why IFT27 protein was more abundant in FT spermatozoa of the GSF group needs to be elucidated. Differences in protein degradation and tyrosine phosphorylation between the fresh pre-freeze and FT spermatozoa are associated with changes in the sperm quality characteristics, such as motility [2,61]. Interestingly, the abundance of IFT27 protein in FT spermatozoa was not associated with improved sperm motility, suggesting that the presence of another cryo-injury mechanism that might be involved in the increased protein levels.

Altogether, the findings of this study indicate that the putative TF binding sites generated by polymorphisms in the 5′-flanking region of *STK35* or *IFT27* gene could impact the process of its regulation. Predictive analysis confirmed that the polymorphic variants in *STK35* and *IFT27* promoters could contribute to differences in the transcriptional activity of the genes, which might predispose spermatozoa to varying susceptibility to cryo-damage. Therefore, we suggest that the allelic variants in *STK35* and *IFT27* could be considered as potential markers for the prediction of boar sperm freezability. In addition, the finding of this study supports future investigations on the effect of *STK35* and *IFT27* promoter polymorphisms on sperm function and survival.

CRediT authorship contribution statement

Anna Mańkowska: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, preparation, Writing – review & editing. **Paweł Brym:** Methodology, Software, Validation. **Przemysław Sobiech:** Formal analysis, Writing – review & editing. **Leyland Fraser:** Methodology, Formal analysis, Investigation, Writing – original draft, preparation, Writing – review & editing, Project administration.

Declaration of competing interest

None of the authors have any conflict of interest to declare.

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Appendix A. Supplementary data

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Paper III

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Expression of *TXNRD1, HSPA4L* and *ATP1B1* Genes Associated with the Freezability of Boar Sperm

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Abstract: Cryopreservation is associated with increased oxidative stress, which is responsible for sperm damage. We analyzed the effect of cryopreservation on mRNA and protein expression of thioredoxin reductase 1 (*TXNRD1*), heat shock protein family A (HSP 70) member 4 like (*HSPA4L*) and sodium/potassium-transporting ATPase subunit beta-1 (*ATP1B1*) genes in boar sperm with different freezability. Boars were classified as having good and poor semen freezability (GSF and PSF, respectively), according to the assessment of post-thaw sperm motility. Total RNA was isolated from fresh pre-freeze (PF) and frozen-thawed (FT) sperm from five boars of the GSF and PSF groups, respectively. Quantification of *TXNRD1*, *HSPA4L* and *ATP1B1* gene expression was performed by RT-qPCR analysis. Proteins extracted from sperm were subjected to Western blotting and SDS-PAGE analyses. Poor freezability ejaculates were characterized by significantly higher relative mRNA expression levels of *TXNRD1* and *HSPA4L* in FT sperm compared with the fresh PF sperm. Furthermore, the relative mRNA expression level of *ATP1B1* was significantly higher in the fresh PF sperm of the GSF group. Western blotting analysis revealed significantly higher relative expression of TXNRD1 protein in the fresh PF sperm of the GSF group, while HSPA4L protein expression was markedly increased in FT sperm of the PSF group. Electrophoretic and densitometric analyses revealed a higher number of proteins in the fresh PF and FT sperm of the PSF and GSF groups, respectively. The results of this study indicate that *ATP1B1* mRNA expression in the fresh PF sperm is a promising cryotolerance marker, while the variations of TXNRD1 and HSPA4L protein expression in the fresh PF or FT sperm provide useful information that may help to elucidate their biological significance in cryo-damage.

Keywords: boar; semen; freezability; stress-related genes; proteins

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1. Introduction

Cryopreservation has been considered to be an efficient method of preserving the genetic materials of different animal species [1,2]. Besides individual variations, cryo-induced oxidative stress has been one of the major factors affecting the post-thaw (PT) quality of boar semen [2–5].

It has been suggested that sperm RNAs are transcriptionally inactive; however, studies have shown that sperm transport specific functional RNAs into the oocyte at the time of fertilization, and these RNAs could contribute to early embryonic development [6–9]. Studies have demonstrated that cryopreservation has varying effects on the profiles of sperm mRNA transcripts, which could be used as markers for sperm functions [8,10–14]. Expression of *PRM1* (Protamine 1) and *PRM2* (Protamine 2), suggested as potential fertility markers, is significantly reduced in frozen-thawed (FT) human sperm [10], and significant changes in the mRNA expression and protein levels of epigenetic-related genes have been

detected in FT boar sperm [11,14]. Evidence has shown that transcriptome modifications in FT bull sperm are associated with reduced fertility [12], and an abundance of differentially expressed (DE) genes that are highly overexpressed in FT bull sperm with poor motility [13].

In this study we selected three stress-related genes, thioredoxin reductase 1 (*TXNRD1*), heat shock protein family A member 4 like (*HSPA4L*) and ATPase Na+/K+ transporting beta-1 polypeptide (*ATP1B1*), which are implicated in various sperm functions, such as spermatogenesis, motility, hyperactivation, capacitation and osmoregulation [15–20]. In boar sperm transcriptome these genes were not differentially expressed (DE) between the good and poor freezability ejaculates [21]. Besides boar sperm transcriptome, *TXNRD1*, *HSPA4L* and *ATP1B1* have been detected in human and ovine sperm transcriptomes [22,23]. The thioredoxin family is implicated in a wide range of cellular processes, including antioxidant defense, signaling of apoptosis, redox control of protein function and transcription factor activity [24,25]. Thioredoxin reductases are a family of selenium-containing pyridine nucleotide–disulfide oxidoreductases that play a key role in redox signaling events [18,24]. Two thioredoxin reductases, TXNRD1 and TXNRD2, have been shown to play a crucial role in maintaining thioredoxins in their reduced (active) state and are components of the cytosolic and mitochondrial thioredoxin systems, respectively [18]. It has been reported that reduced TXNRD1 content in asthenozoospermic sperm is associated with increased reactive oxygen species (ROS) generation, apoptosis and the number of immature sperm [26]. Moreover, HSPA4L is a member of the heat shock protein family (HSP110) that performs many functions, such as molecular chaperones that are synthesized under stress conditions to assist in protein folding, assembly and transport [15,16,27]. Evidence has shown that HSPA4L is required for normal spermatogenesis, and is produced under environmental stress and pathological or certain physiological conditions to protect cells from damage [15,16]. The HSPA4L protein is highly expressed in different spermatogenic cells and appears to play a role in osmotolerance, male fertility and sperm motility [15,23]. The protein encoded by the *ATP1B1* gene belongs to the family of Na+/K+–ATPase pumps, a mechanism responsible for maintaining the electrochemical gradient ions across the cellular membrane that could be disturbed by osmotic-induced stress [17,28,29]. The ATP1B1 protein has been detected in epididymal sperm of bulls and swamp buffalos [30,31], and its significantly high expression in bull epididymal sperm was associated with good semen freezability [30,31].

In this study, we hypothesized that the differential expression of the stress-related genes (*TXNRD1*, *HSPA4L* and *ATP1B1*) in boar sperm might be associated with cryotolerance. The main objective of the study was to determine the mRNA and protein expression levels of *TXNRD1*, *HSPA4L* and *ATP1B1* in fresh pre-freeze (PF) and FT sperm from boars classified as having good and poor semen freezability (GSF and PSF, respectively). In addition, the identification of the electrophoretic profiles of sperm proteins associated with freezability was performed in this study.

2. Results

2.1. Semen Quality Assessment

The quality characteristics of the fresh PF semen did not differ ($p > 0.05$) between the GSF and PSF groups (Table 1). ANOVA results showed significant boar effects ($p < 0.05$) on the quality characteristics of FT sperm (Supplementary Table S1). We classified five boars each of the GSF (Boars no. 1–5) and PSF groups (Boars no. 6–10), according to post-thaw total motility (TMOT). Boars showing more than 30% (>30%) post-thaw TMOT were assigned to the GSF group, whereas those with TMOT less than 30% (<30%) were allocated to the PSF group (Table 2).

Besides post-thaw TMOT and progressive motility (PMOT), motion parameters analyzed by the computer-assisted sperm analysis (CASA) system showed higher ($p < 0.05$) PT values of velocity straight line (VSL), velocity average path (VAP) and velocity curvilinear (VCL), straightness (STR) and linearity (LIN) for the GSF group than the PSF group (Table 2). Likewise, the percentages of FT sperm with mitochondrial membrane potential

(MMP), plasma membrane integrity (PMI), NAR acrosome integrity and DNA integrity were significantly higher ($p < 0.05$) in the GSF group than in the PSF group (Table 2).

Table 1. Characteristics of fresh pre-freeze (PF) boar sperm prior to cryopreservation. Values are expressed as the mean ± SEM. GSF—good semen freezability; PSF—poor semen freezability.

Sperm Parameters	GSF (n = 25)	PSF (n = 25)	p-Value
Total motility (TMOT, %)	89.39 ± 0.69	87.38 ± 0.92	0.846
Progressive motility (PMOT, %)	63.84 ± 1.24	65.83 ± 1.06	0.231
Velocity straight line (VSL, μm/s)	75.90 ± 2.10	77.18 ± 2.01	0.584
Velocity average path (VAP, μm/s)	91.90 ± 1.76	93.58 ± 2.46	0.767
Velocity curvilinear (VCL, μm/s)	127.18 ± 4.20	125.18 ± 4.20	0.882
Straightness (STR, %)	81.35 ± 1.85	82.73 ± 1.29	0.535
Linearity (LIN, %)	60.32 ± 1.35	62.16 ± 1.45	0.355
Amplitude of lateral head displacement (ALH, μm)	7.16 ± 0.24	7.44 ± 0.19	0.657
Beat cross frequency (BCF, Hz)	30.40 ± 0.72	31.83 ± 0.95	0.231
Mitochondrial membrane potential (MMP, %)	87.51 ± 0.56	88.14 ± 0.67	0.664
Plasma membrane integrity (PMI, %)	88.17 ± 0.51	87.55 ± 1.05	0.721
NAR acrosome integrity (%)	91.88 ± 0.49	90.76 ± 0.71	0.185
DNA fragmentation (%)	2.23 ± 0.30	2.38 ± 0.26	0.698

Differences between the GSF and PSF groups for VSL, VAP, VCL, ALH and BCF parameters were compared, using the Mann–Whitney U test. Significant at $p < 0.05$; NAR—normal apical ridge.

Table 2. Post-thaw (PT) characteristics of boar sperm. Values are expressed as the mean ± SEM. GSF—good semen freezability; PSF—poor semen freezability.

Sperm Parameters	GSF (n = 25)	PSF (n = 25)	p-Value
Total motility (TMOT, %)	51.29 ± 1.47 [a]	24.66 ± 0.82 [b]	0.001
Progressive motility (PMOT, %)	40.71 ± 1.28 [a]	17.17 ± 1.35 [b]	0.001
Velocity straight line (VSL, μm/s)	60.97 ± 2.39 [a]	46.15 ± 3.02 [b]	0.001
Velocity average path (VAP, μm/s)	76.84 ± 2.56 [a]	60.48 ± 3.16 [b]	0.001
Velocity curvilinear (VCL, μm/s)	133.12 ± 3.50 [a]	112.18 ± 2.79 [b]	0.001
Straightness (STR, %)	79.10 ± 1.22 [a]	75.20 ± 1.43 [b]	0.042
Linearity (LIN, %)	46.07 ± 1.56 [a]	40.75 ± 1.18 [b]	0.049
Amplitude of lateral head displacement (ALH, μm)	5.49 ± 0.27 [a]	6.42 ± 1.09 [a]	0.375
Beat cross frequency (BCF, Hz)	21.70 ± 0.96 [a]	28.23 ± 3.12 [a]	0.072
Mitochondrial membrane potential (MMP, %)	52.10 ± 1.24 [a]	31.61 ± 1.64 [b]	0.001
Plasma membrane integrity (PMI, %)	51.24 ± 1.09 [a]	36.46 ± 1.26 [b]	0.001
NAR acrosome integrity (%)	51.45 ± 1.34 [a]	38.50 ± 1.58 [b]	0.001
DNA fragmentation (%)	7.05 ± 0.48 [a]	12.90 ± 0.67 [b]	0.001

Differences between the GSF and PSF groups for VSL, VAP, VCL, ALH and BCF parameters were compared, using the Mann–Whitney U test. Values with different letters (a and b) within the same row are significantly differed ($p < 0.05$); NAR—normal apical ridge.

2.2. Analysis of Gene Expression

There were wide variations in the mRNA expression levels of *TXNRD1*, *HSPA4L* and *ATP1B1* in the fresh PF or FT sperm among the boars. It was found that there were significant effects on *TXNRD1* mRNA expression among the groups (Kruskal–Wallis ANOVA,

df = 3, $p < 0.011$). FT sperm exhibited significantly higher ($p < 0.05$) *TXNRD1* mRNA expression compared with the fresh PF sperm of the PSF group (Figure 1A). In contrast, no significant differences ($p > 0.05$) were found between the fresh PF and FT sperm of the GSF group (Figure 1A). Significant variations in *HSPA4L* mRNA expression were observed among the groups (Kruskal–Wallis ANOVA, *df* = 3, $p < 0.012$). *HSPA4L* mRNA expression levels in FT sperm of the GSF and PSF groups were significantly higher ($p < 0.05$, respectively) than in the fresh PF sperm of the PSF group (Figure 1B). Significant treatment effects on *ATP1B1* mRNA expression levels were observed among the groups (Kruskal–Wallis ANOVA, *df* = 3, $p < 0.047$). Furthermore, the fresh PF sperm of GSF group exhibited approximately 3-fold higher ($p < 0.05$) *ATP1B1* mRNA expression compared with the PSF group (Figure 1C).

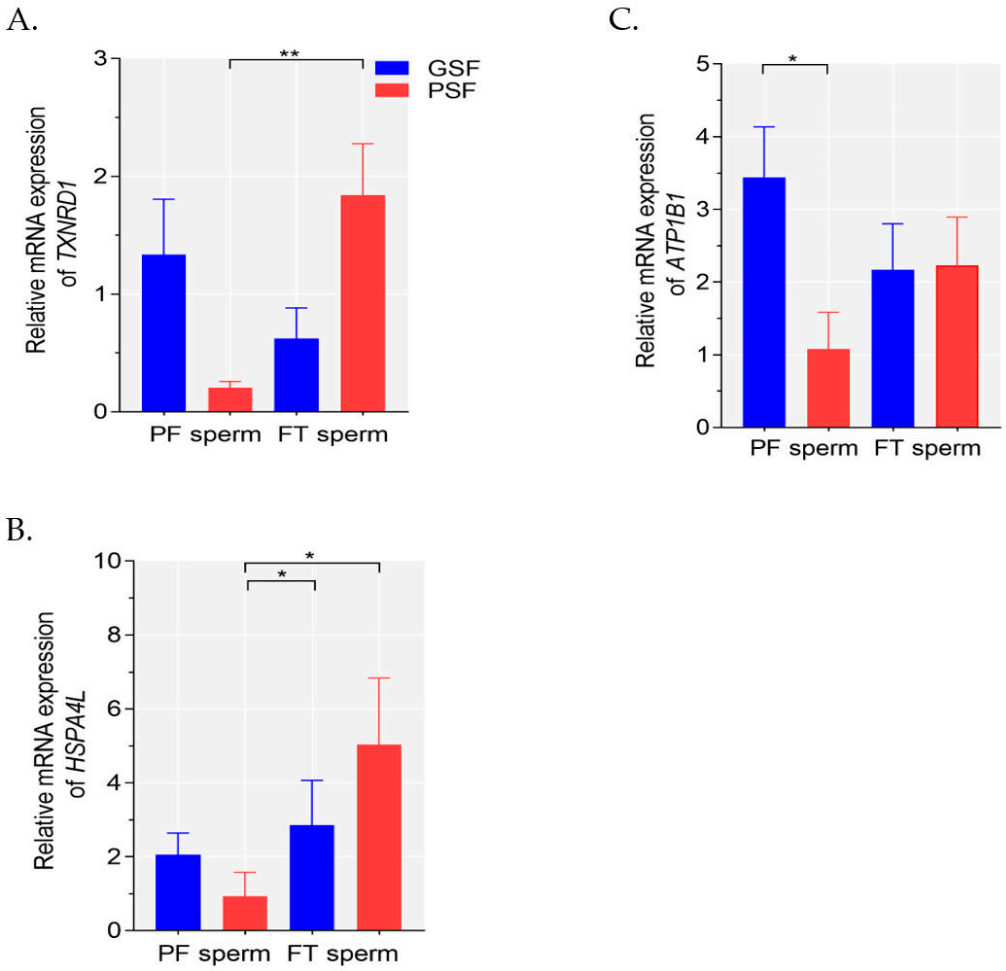


Figure 1. Relative mRNA expression of (**A**) *TXNRD1*, (**B**) *HSPA4L* and (**C**) *ATP1B1* in fresh pre-freeze (PF) and frozen-thawed (FT) sperm from boars of the good and poor semen freezability (GSF and PSF, respectively) groups. Values are presented as the mean ± SEM. Expression values of *TXNRD1*, *HSPA4L* and *ATP1B1* were normalized against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) value of the control. Significant at * $p < 0.05$, and ** $p < 0.01$ after the Bonferroni correction.

2.3. Gene Ontology (GO) Enrichment Analysis and KEGG Pathways

The GO mitochondrial function (GO:MF) terms for *TXNRD1* and *ATP1B1* were represented by glutathione oxidoreductase activity (GO:0097573) and ATPase activator activity (GO:0001671) (Supplementary Table S2A). The GO biological process (GO:BP) term for *HSPA4L* was represented by novo centriole assembly (GO:0097742), while the GO:BP term for *TXNRD1* and *ATP1B1* was represented by cellular homeostasis (GO:0019725) (Supplementary Table S2B). Most of the GO cellular components GO:CC terms were

represented by *ATP1B1*, such as ATPase-dependent transmembrane transport complex (GO:0098533) (Supplementary Table S2C). *TXNRD1*, *HSPA4L* and *ATP1B1* were assigned to different Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, such as selenocompound metabolism (KEGG:00450), FoxO signaling pathway (KEGG:04068) and thyroid hormone synthesis (KEGG:04918), respectively (Supplementary Table S2D).

2.4. Western Blotting Analysis

Western blotting analysis showed significant variations in the expression of TXNRD1 (Kruskal–Wallis ANOVA, $df = 3$, $p < 0.002$), HSPA4L (Kruskal–Wallis ANOVA, $df = 3$, $p < 0.008$) and ATP1B1 (Kruskal–Wallis ANOVA, $df = 3$, $p < 0.001$) proteins among the groups. Blots showing TXNRD1, HSPA4L and ATP1B1 protein expression in the fresh PF and FT sperm are indicated in Figures 2A and 2B, respectively.

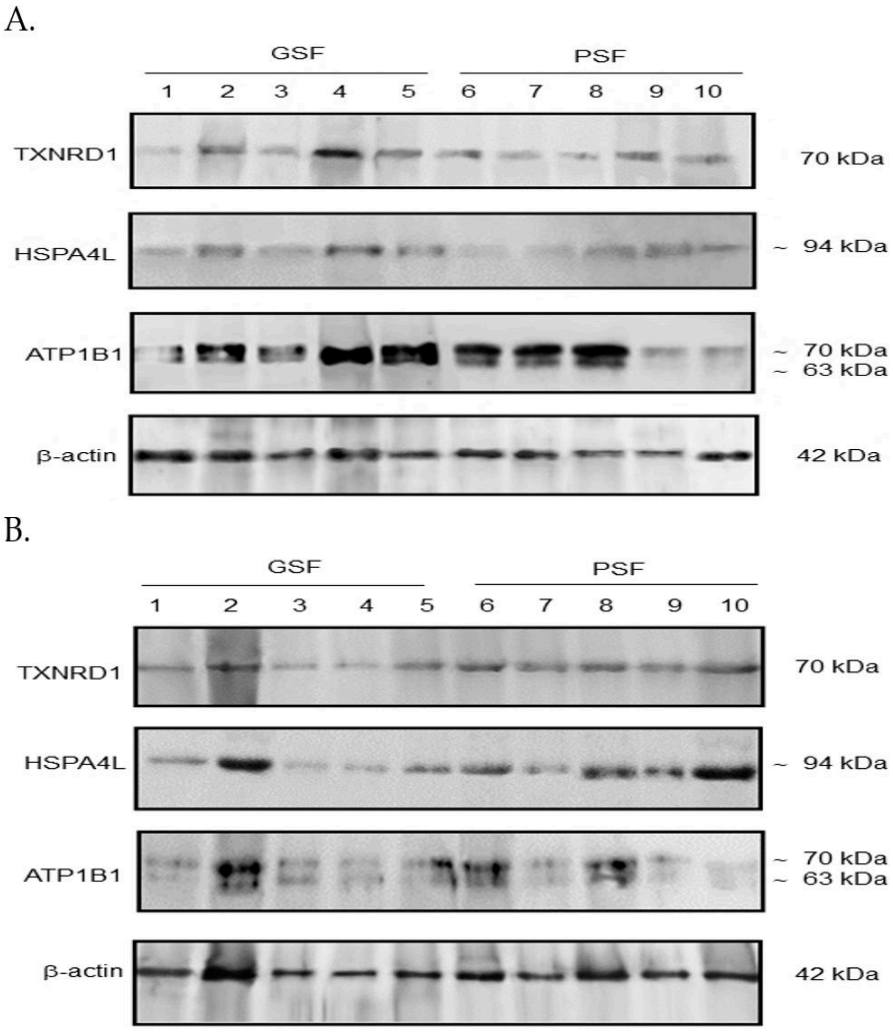


Figure 2. Western blotting analysis showing protein expression of TXNRD1, HSPA4L and ATP1B1 in (**A**) the fresh pre-freeze (PF) and (**B**) frozen-thawed (FT) sperm from boars of the good and poor semen freezability (GSF and PSF, respectively) groups.

Bands representing 70 kDa for TXNRD1, and approximately 94 kDa (~94 kDa) for HSPA4L were detected in the lysates either from the fresh PF sperm (Figure 2A) or FT sperm (Figure 2B). We detected two protein bands (with molecular weights ~63 kDa and ~70 kDa) for ATP1B1 in the fresh PF and FT sperm (Figures 2A and 2B, respectively). Analysis showed variations in the expression levels of the analyzed proteins in the PF and FT sperm among boars (Figures 2A and 2B, respectively).

The fresh PF and FT sperm of the PSF group exhibited relatively higher ($p < 0.05$ and $p < 0.01$, respectively) TXNRD1 protein expression compared with the fresh PF of the GSF group (Figure 3A). It was found that the FT sperm of the GSF and PSF groups exhibited higher ($p < 0.05$ and $p < 0.01$, respectively) HSPA4L protein expression than the fresh PF sperm of the PSF group (Figure 3B). Furthermore, the fresh PF sperm of the GSF and PSF groups exhibited relatively higher ($p < 0.01$ and $p < 0.05$, respectively) expression of ATP1B1 protein (Figure 3C).

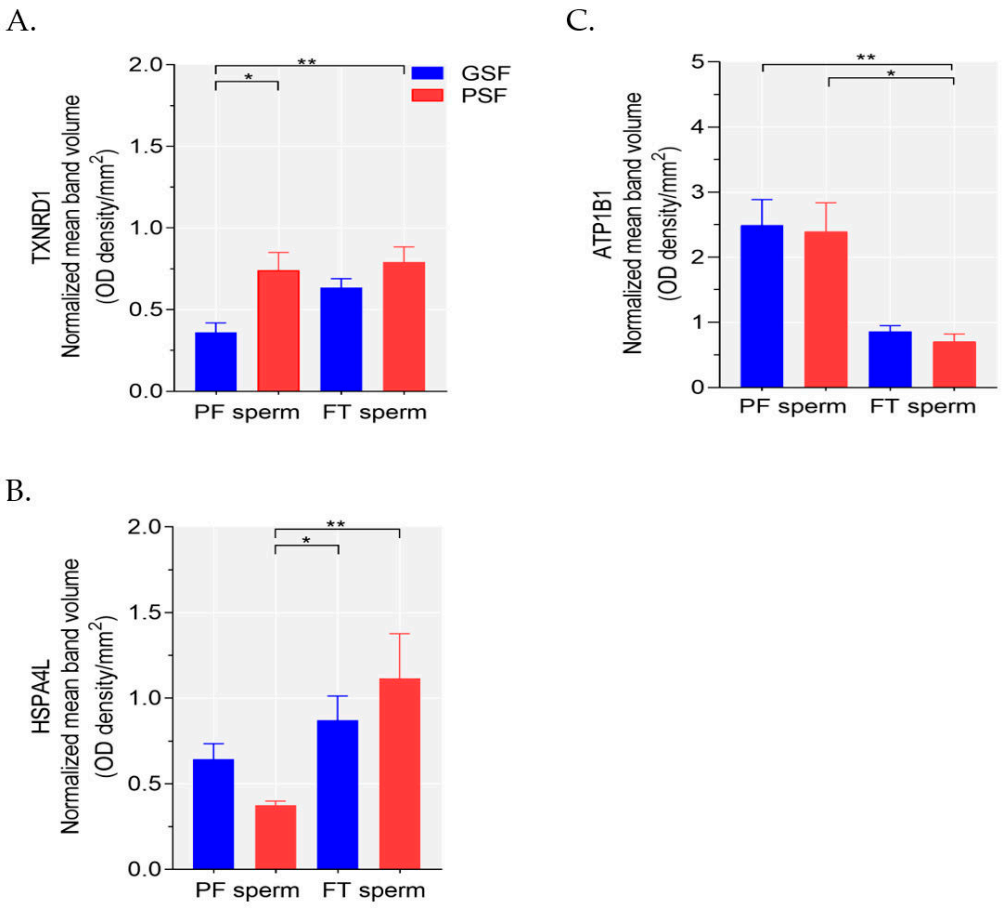


Figure 3. Relative expression of (**A**) TXNRD1, (**B**) HSPA4L and (**C**) ATP1B1 proteins in the fresh pre-freeze (PF) and frozen-thawed (FT) sperm from boars of the good and poor semen freezability (GSF and PSF, respectively) groups. Values represent the mean ± SEM. β-actin was used as the control to normalize the relative expression of the analyzed proteins. Significant at * $p < 0.05$, and ** $p < 0.01$ after the Bonferroni correction.

2.5. Protein–Protein Interaction (PPI) Networks

The predicted PPI networks, generated by the STRING database v11.5, showed that TXNRD1, HSPA4L and ATP1B could interact and co-regulate the functions of different proteins involved in various biological processes (Figure 4A–C). Color saturation of the edges represented both functional and physical protein associations. A total of 21 nodes and 174 edges were identified for TXNRD1 (Figure 4A). Furthermore, a total of 21 nodes and 133 edges were constructed for HSPA4L, which comprised five BAG (Bcl-2 associated athanogene) cochaperones (Figure 4B). The network-based associations showed that the ATP1B1 protein was constructed with 21 nodes and 113 edges, and interacted with nine different proteins of the glycoprotein subunits of Na+/K+–ATPase (Figure 4C).

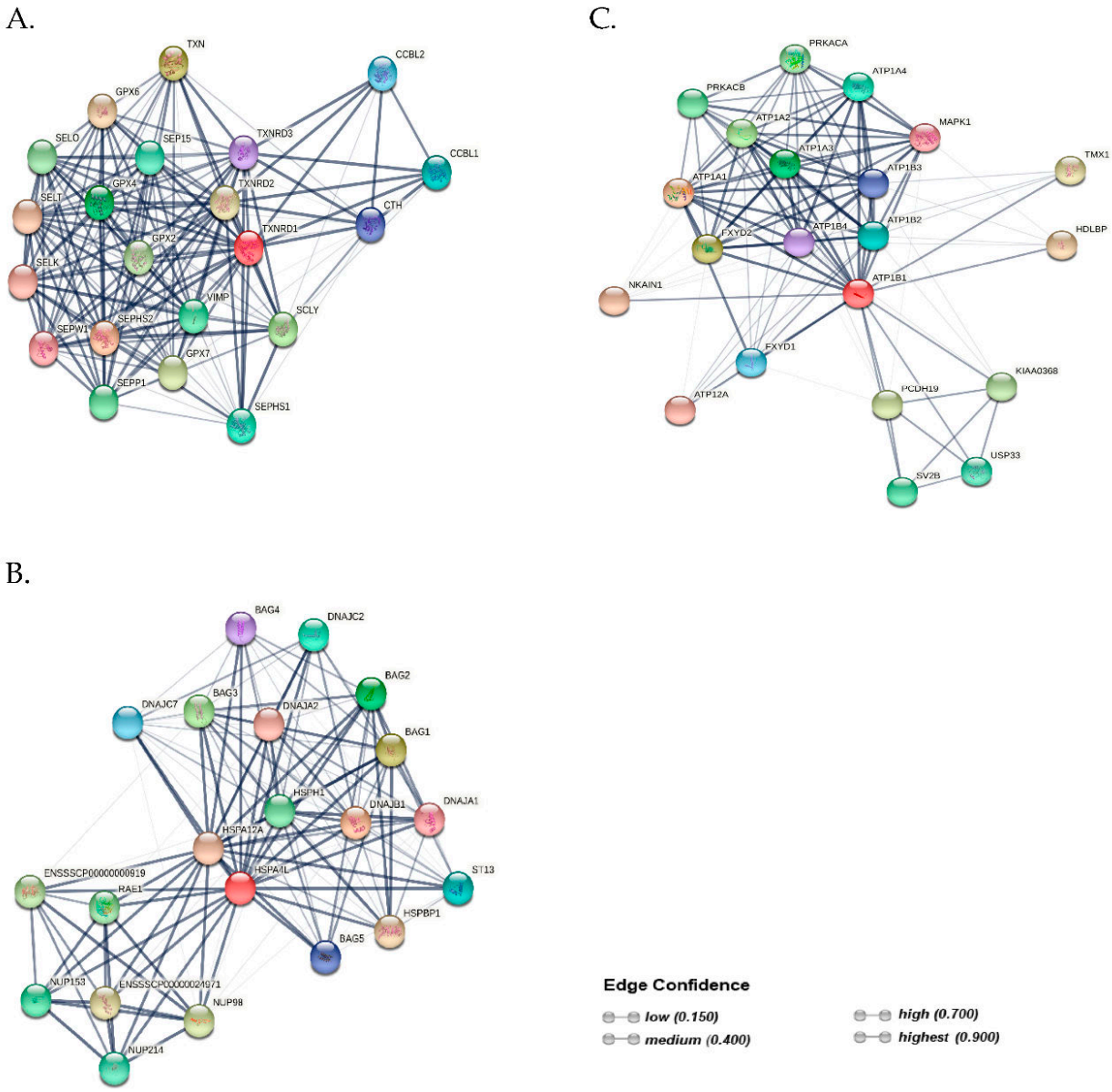


Figure 4. Protein–protein interaction (PPI) network visualized by STRING software. The nodes indicate proteins, and edges indicate the number of interactions for (**A**) TXNRD1, (**B**) HSPA4L and (**C**) ATP1B1.

2.6. SDS-PAGE Analysis

In most of the boars, electrophoretic and densitometric analyses showed variations in the protein profiles between the fresh PF and FT sperm (Supplementary Figure S1). Protein abundance was more marked in the fresh PF sperm of the PSF group compared with the GSF group (89 vs. 65, respectively), while the FT sperm of the GSF group revealed a higher number of proteins (85 and 68, respectively), as shown in Figure 5. In general, cryopreservation resulted in the appearance of 40 and 16 additional proteins in the GSF (Supplementary Figure S1A) and PSF groups (Supplementary Figure S1B), respectively. The electrophoretic profiles of the FT sperm of three boars (Boars no. 1–3) showed the appearance of ten to eleven additional proteins, with molecular weights ranging from 14 to 137 kDa, while seven protein bands of 15–117 kDa were detected in the FT sperm of Boar no. 4 (Supplementary Figure S1A). Furthermore, the SDS-PAGE profiles of the FT sperm from one boar of the PSF group (Boar no. 9) showed the appearance of six additional protein bands of 17–103 kDa (Supplementary Figure S1B).

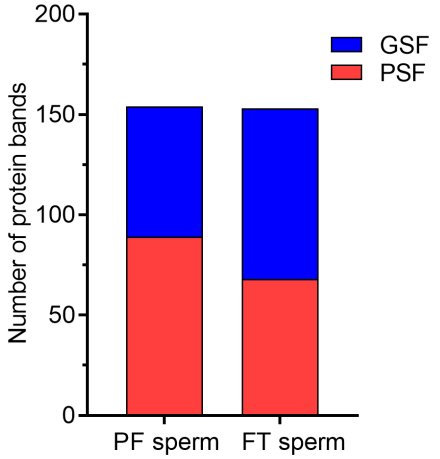


Figure 5. The average number of protein bands in the fresh pre-freeze (PF) and frozen-thawed (FT) sperm following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry analyses.

In general, the SDS-PAGE and densitometric analyses showed variations in the distributions of the protein fractions among the boars (Figure 6A,B). Most of the boars of the GSF group showed a higher distribution (27.0–28.6%) of proteins of 21–40 kDa (21–40 kDa) in either the fresh PF or FT sperm (Figure 6A). Likewise, proteins with molecular weights lower than 20 kDa (<20 kDa) revealed a higher distribution (21.4–30.7%) in the fresh PF or FT sperm from three boars of the PSF group (Figure 6B).

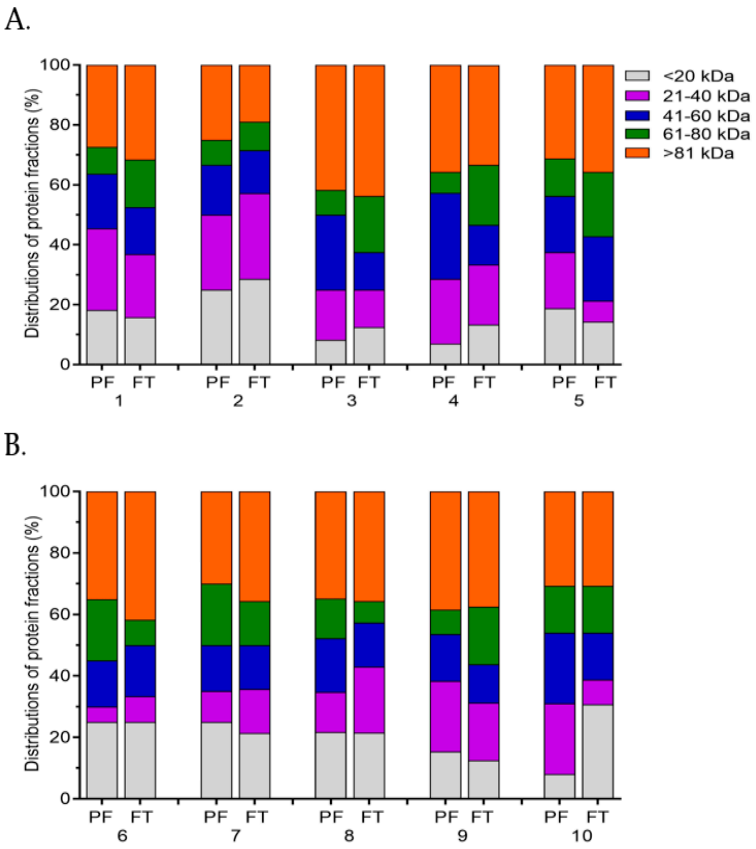


Figure 6. Distributions of the protein fractions in the fresh pre-freeze (PF) and frozen-thawed (FT) sperm from boars differing in freezability. (**A**) Protein fractions in the fresh PF and FT sperm from boars of the good semen freezability (GSF) group (Boars no. 1–5). (**B**) Protein fractions in the fresh PF and FT sperm from boars of the poor semen freezability (PSF) group (Boars no. 6–10).

It was found that Boars nos. 3 and 4 showed approximately 2-fold higher distribution of proteins of 41–60 kDa (41–60 kDa) and 61–80 kDa (61–80 kDa) in the fresh PF and FT sperm, respectively (Figure 6A). Variations in the protein distribution groups of 41–60 kDa and 61–80 kDa among the boars of the PSF group were less marked between the fresh PF and FT sperm (Figure 6B). Also, the distribution of the proteins with molecular weight higher than 81 kDa (>81 kDa) was not markedly different between the fresh PF and FT sperm, regardless of the freezability group (Figure 6A,B).

3. Discussion

To the best of our knowledge, this study is the first to demonstrate that cryopreservation has varying effects on the relative mRNA expression levels of *TXNRD1*, *HSPA4L* and *ATP1B1* in boar sperm. Unexpectedly, while there were no marked differences in the mRNA expression of *TXNRD1* between the fresh PF and FT sperm of the GSF group, significantly higher expression of the gene was detected in FT sperm of the PSF group. Although the underlying mechanism responsible for high post-thaw mRNA *TXNRD1* expression in poor freezability ejaculates is not yet fully understood, we hypothesize that this phenomenon might reflect increased oxidative stress, which could reduce the sperm cryotolerance. Accordingly, *TXNRD1* is implicated in the maintenance of the redox state of oxidized proteins by scavenging ROS, thereby protecting sperm cells against oxidative damage [18]. However, the mechanisms responsible for the increased content of *TXNRD1* mRNA in FT sperm from poor freezability ejaculates remain unclear and would require further investigation.

Similar to mRNA *TXNRD1* expression, significantly higher mRNA expression of *HSPA4L* was detected in FT sperm of the PSF group. Evidence has shown that HSPA4L functions as a molecular chaperone and provides protection against oxidative stress in sperm [15,16]. Transcriptome analysis of FT bull sperm detected the up-regulation of four DE genes, such as ribosomal protein L31 (*RPLC*), glutamate-cysteine ligase catalytic subunit (*GCLC*), protein kinase C epsilon type (*PRKCE*) and proteolipid protein 1 (*PLP1*); however, the up-regulation of *GCLC* was suggested to be a protective response of the sperm to cold shock and oxidative stress incurred following freezing-thawing [12]. Moreover, transcriptome profiling of FT bull sperm revealed that transcripts regulating reactive oxygen species (ROS) production and maintenance of the physiological function of the cytoskeletal proteins were highly expressed in ejaculates with poor motility compared with those characterized by good motility [13]. Furthermore, the expression levels of genes encoding cold shock protein A (*CspA*), heat shock protein 60 (*HSP60*) and heat shock protein 10 (*HSP10*) were significantly increased in FT bull sperm [32]. Moreover, it has been demonstrated that cryopreservation induced alterations in the mRNA expression of epigenetic-related genes, resulting in the compromised functions of FT boar sperm [14]; however, it has been suggested that methylation modifications might not be the main factors responsible for increased mRNA expression of heat shock protein 70 (*HSP70*) and heat shock protein 90 (*HSP90*) in FT sperm [33]. Also, in the case of bull sperm transcriptome it was suggested that the abundant expression of transcript cytochrome oxidase subunit 7C (*COX7C*) in FT semen from low-fertility bulls might be due to the inefficient translation of the transcript [34]. In another study, transcriptome analysis of FT boar sperm showed that a marked increase in the expression of several genes that was associated with the signaling pathways, such as phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) and cAMP signaling pathways [35]. Despite accumulating evidence, the mechanisms underlying the increase in mRNA expression levels of sperm-borne genes in FT semen from different animal species are unclear. In previous studies, it has been suggested that cryopreservation facilitated disturbance in the mRNA–protein interactions, thus rendering the mRNA molecules more susceptible to cryo-induced damage [10,33]. More importantly, the degradation of mRNA during the freezing-thawing procedure has been suggested as one of the factors that might be responsible for the reduced gene expression levels in FT sperm, because mature sperm probably lack active transcriptional machinery and do not have

the ability to replace mRNA that is lost following cryopreservation [10,36]. More recently, we have confirmed that sperm from the poor freezability ejaculates are predisposed to increased oxidative stress [21]; however, it is unclear at this point whether higher *TXNRD1* or *HSPA4L* mRNA relative levels in FT sperm of the PSF group might be a protective response against cryo-induced oxidative stress. The mechanisms underlying the variations in mRNA expression levels of either gene between the fresh PF and FT sperm remain to be fully explored.

It is apparent that the higher mRNA expression of *HSPA4L* in FT sperm was accompanied by increased protein expression, regardless of the freezability group. The findings of the current study are not consistent with those of other studies, which have shown that low protein expression of HSPA4L was correlated with poor human sperm quality [15,16]. Interestingly, the response of sperm of both freezability groups to increased cryo-induced oxidative stress was accompanied by an abundance of HSPA4L protein, which did not contribute to an improvement in FT sperm quality, particularly in the PSF group. Similar observations have been reported in a recent study, which has shown that the abundance of HSP70 protein in FT water buffalo sperm was not accompanied by improved sperm functions, but rather by capacitation-like events, probably resulting in the synthesis of the chaperone protein [37]. Accumulating evidence has shown that oxidative stress, induced by ROS production following cryopreservation, compromises the sperm fertilizing ability [2–4,31]. It has been suggested that heat shock proteins are activated during stressful conditions in order to stimulate a pro-survival response of sperm cells during oxidative damage [16]. Accumulating evidence has shown that proteins that are involved in regulating stress-induced ROS production are more abundant in FT sperm [12,38,39]. Based on the findings of the current study, the abundance of the TXNRD1 and HSPA4L proteins in FT sperm did not provide protection against sperm cryo-damage, even though TXNRD1 and HSPA4L are closely associated with antioxidant protection (Figure 4A) and apoptotic rate (Figure 4B), respectively. The precise mechanism responsible for this phenomenon is currently unclear and warrants further investigation.

ATP1B1 is an integral membrane protein that is involved in the regulation and maintenance of the electrochemical gradient of Na+ and K+ ions across the plasma membrane [28,29]. The functional Na+/K+–ATPase consists of an alpha subunit (110 kDa) and a beta subunit comprising 35–60 kDa, depending on glycosylation [17,28,29]. More importantly, ATP1B1 is one of the subunits of Na+/K+–ATPase that is regulated by the complex assembly of alpha/beta heterodimer (Figure 4C). Moreover, ATP1B1 is the subunit of ATP synthase that must undergo a conformational change to obtain energy, and its inactivation can affect various sperm functions, such as mitochondrial activity and motility [40]. Interestingly, the results of the current study show that high *ATP1B1* mRNA relative levels in the fresh PF sperm of the GSF group were not accompanied by an increase in sperm motility compared with the PSF group (Table 1). We suggest that the effect of mRNA *ATP1B1* expression in poor freezability ejaculates on the sperm functions is poorly understood. However, it is worth stressing that higher *ATP1B1* mRNA expression in the fresh PF sperm of the GSF group is consistent with the findings of our previous study, which confirmed that good freezability ejaculates exhibited high levels of NADH dehydrogenase subunit 6 (*ND6*) expression, a key component of the oxidative phosphorylation (OXPHOS) complex that is the most important pathway for ATP production [21]. We speculate that the reduced *ATP1B1* mRNA expression in the fresh PF semen of the PSF group could predispose sperm to reduced cryotolerance. Furthermore, we did not detect marked differences in the ATP1B1 protein levels in the fresh PF sperm between the freezability groups. The findings of this study are not consistent with those of a previous study, which demonstrated that increased post-thaw motility and mitochondrial function of epididymal sperm from the good freezability ejaculates were associated with higher expression of the ATP1B1 protein [30]. In the current study, we detected two protein bands of ATP1B1 (~63 kDa and ~70 kDa); however, we are not in a position to explain whether these isoforms could have resulted from glycosylation, even though a previous study

reported the presence of glycosylated isoforms of ATP1B1 in the head membrane of bull sperm [17]. Furthermore, it appears that the significant loss of ATP1B1 protein in FT sperm of the PSF group indicates marked membrane damage, thus reiterating our findings that the poor freezability ejaculates are characterized by reduced cryo-damage.

It is noteworthy that the mRNA expression of *TXNRD1* and *ATP1B1* was not concurrent with the protein abundance, particularly in FT sperm. However, evidence has indicated that, as a result of post-translational modifications in mature sperm, the abundance of proteins does not always reflect their relative expression levels of mRNA transcripts [7,41,42]. Presently, the mechanisms responsible for the variations in protein expression between the fresh PF and FT sperm have not been fully elucidated. However, several authors have suggested that such phenomena could be due to several factors, such as inefficient translation, post-translational modifications, enhanced phosphorylation or capacitation-like events [34,37,41,43,44]. According to Bogle et al. [45], variations in protein abundance between the fresh PF and FT sperm could be the consequence of a combination of protein degradation, post-translational processing, and alterations in the secondary or tertiary structures and/or translocation to other structures or outside the sperm cell. We do not know whether such phenomena could be a possible explanation for the abundance of TXNRD1 and HSPA4L proteins, as well as the electrophoretic protein fractions in FT sperm. Previous studies have demonstrated that the expression of several proteins that are involved in sperm capacitation, signal transduction, response to stress, energy status and sperm-oocyte fusion was increased in FT semen of boars, humans and rams [38,45,46]. In our previous study, we demonstrated that increased oxidative stress was triggered by the up-regulation of inflammatory and apoptosis-related genes in sperm from boars with low cryotolerance [21]. It should be emphasized that the abundance of TXNRD1 protein in the fresh PF sperm of the PSF group indicates that ejaculated sperm are predisposed to increased oxidative damage, which has been shown to be a characteristic feature of the poor freezability ejaculates [21]. However, this finding was not consistent with the expression levels of HSPA4L protein in the fresh PF sperm of the PSF group, suggesting that the chaperone protein might be responding to different stimuli [47]. In the current study, the lack of significant differences between the freezability groups regarding the post-thaw expression levels of TXNRD1, HSPA4l or ATP1B1 protein remains unclear, and suggests the need for further research to explain these findings. Even though our findings show marked differences in the electrophoretic protein profiles in the fresh PF sperm or PT sperm between the GSF and PSF groups, we did not analyze the association of the protein bands with sperm freezability. Moreover, a previous study has reported on the effects of proteins with different molecular weights on the cryotolerance of boar sperm [48]. According to Corcini et al. [48], proteins with molecular weights of 18, 19, 44, 65, 80 and 100 kDa could be potential markers for the sperm tolerance to the freezing-thawing procedure. Other studies have reported that specific sperm proteins were associated with good semen freezability and fertility [49,50]. It can be suggested that alterations in the protein profiles of FT sperm might lead directly to reduced fertility. We suggest that further studies will be needed to explain the significance of the protein fractions in the cryotolerance of boar sperm.

4. Materials and Methods

4.1. Chemicals and Media

Chemicals were bought from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

4.2. Animals and Semen Collections

Ten Polish large white (PLW) boars (with an average age of two years) were used in this study.

The boars were stationed at the Cryopreservation laboratory, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn. A total of five ejaculates were collected from each boar during the autumn–winter period, using the gloved-hand

technique [21]. Water was available ad libitum. Semen samples with more than 70% sperm motility and 85% morphologically normal sperm were used in the study. All animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee in Olsztyn (Poland). Approval of the Local Ethics Committee for experiments on boars (semen collection procedure) has not been required since 15 January 2015.

4.3. Semen Processing Procedure

Semen was frozen using lyophilized lipoprotein fractions of ostrich egg yolk (LPFo) according to a cryopreservation protocol [3,51,52]. Following the cooling of extended semen (2 h at 5 °C), samples were diluted (2:1) with a freezing extender (89.5 mL lactose-LPFo extender, 9 mL glycerol and 1.5 mL Orvus Es Paste), before being packaged in 10-mL sterilized aluminum tubes (500 × 10^6 spermatozoa/mL). Samples were frozen in a programmable controlled-rate freezer (Ice Cube 1810, SY-LAB, Austria), using an appropriate cooling rate [51]. Prior to PT analysis, the samples were thawed in a water bath for 60 s at 50 °C, diluted in Beltsville Thawing Solution (BTS) and held in a water bath for 10 min at 37 °C.

4.4. Semen Quality Assessment

Assessment of quality characteristics was performed on the fresh PF and FT sperm.

4.4.1. CASA Motility and Motion Parameters

Sperm motility and motion parameters were assessed with the CASA system (HTR-IVOS 12.3, Hamilton Thorne Biosciences, MA, USA) using the software settings described in a previous study [21]. The sperm parameters assessed by the CASA system included TMOT (%), PMOT (%), VSL (μm/s), VAP (μm/s), VCL (μm/s), STR (ratio of VSL/VAP × 100%), LIN (ratio of VSL/VCL × 100%), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz).

4.4.2. Membrane Integrity Characteristics

The percentage of sperm with functional MMP was assessed with the fluorescent lipophilic cation JC-1 and propidium iodide (PI) fluorescent dyes [53]. Sperm samples were diluted (30 × 10^6 spermatozoa/mL) in a HEPES-buffered solution, and incubated with JC-1 and PI solutions at 37 °C. A minimum of 100 cells per slide were examined at ×600 magnification under a fluorescence microscope (Olympus CH 30, Olympus Optical Co. Ltd. Tokyo, Japan), and sperm that exhibited orange–red fluorescence in the midpiece region were considered as viable sperm cells with functional mitochondria. Slides were analyzed in duplicate.

The percentage of sperm with PMI was assessed with the SYBR-14 and PI fluorescent probes, using the Live/Dead Sperm Viability Kit [54]. A minimum of 100 cells per slide were examined at ×600 magnification under a fluorescence microscope (Olympus CH 30), and two slides were evaluated per sample.

A modified Giemsa staining protocol was used to assess the percentage of sperm with NAR acrosome integrity [55,56]. Briefly, smears made on slides were fixed by immersion in formal saline solution before being stained with the Giemsa staining solution for 90 min at room temperature, prior to analysis. A minimum of 100 cells per slide, two slides per sample, were examined under a bright light microscope, equipped with oil-immersion lens at ×1000 magnification (Olympus BX 41, Olympus, Tokyo, Japan), and were classified as sperm with intact apical ridge or damaged apical ridge.

4.4.3. DNA Fragmentation

Sperm DNA fragmentation was assessed, using the comet assay [3,51]. Briefly, semen samples were washed by centrifugation (800× *g*, 5 min) and the pellets (10 × 10^6 spermatozoa/mL) were re-suspended in a phosphate-buffered solution (PBS). Samples were placed on frosted microscope slides pre-coated with normal melting point agarose for lysing and RNase A

treatment. Following treatment, samples were subjected to electrophoresis, before being fixed in 70% ethanol and stained with ethidium bromide. A minimum of 200 cells per slide were examined in random fields at 400× magnification under a fluorescence microscope (Olympus BX 41, Olympus, Tokyo, Japan), and sperm were classified as non-fragmented DNA (undamaged) and fragmented DNA (damaged) sperm cells. Slides were analyzed in duplicate.

4.5. Total RNA Isolation and Quantitative Real-Time PCR (RT-qPCR) Analysis

Total RNA was isolated from the fresh PF and FT sperm from ten boars, according to a previously described method [21]. At least three sperm samples (n = 3) from each boar were used for RNA isolation. Briefly, fresh PF and FT sperm samples (150 × 10^6 spermatozoa/mL) were washed (5000× g for 5 min at 4 °C) in PBS, and sperm pellets were re-suspended in a hypotonic solution supplemented with 0.5% Triton X-100, prior to storage on ice for 20 min. Following this, samples were washed 2× in PBS, and were treated with a Lysis Buffer (PureLink RNA mini kit). The RNA extraction protocol was based on the utilization of the TRIzol/Pure Link™ RNA Mini kit (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA), in conjunction with a Turbo DNase digestion procedure. The quantity, purity and 260/280 nm ratios of total RNA were examined by a Nanodrop Spectrophotometer (ND-1000, NanoDrop, Thermo Fisher Scientific Inc., Waltham, MA, USA), as described in a previous study [21].

The relative mRNA quantification of *TXNRD1*, *HSPA4L* and *ATP1B1* was performed using RT-qPCR analysis, according to a previously described method [57], with some modifications. The RNA samples were denatured for 10 min at 70 °C before the synthesis of cDNA. Two hundred nanograms of RNA were used as a template, and the reactions were performed using the High Fidelity cDNA Synthesis Kit (Roche Diagnostics International, Basel, Switzerland) with random hexamer, according to the manufacturer's protocol. The cDNA synthesis was performed in a PCR Thermal Cycler (Labcycler, Sensoquest GmbH, Göttingen, Germany). All analyses were performed in duplicates. Aliquots of the RT products were diluted with nuclear-free water and used for RT-PCR analysis.

Amplifications were performed in a Real-Time PCR system (ABI 7900 HT, Applied Biosystems, Foster City, CA, USA) using the master mix volume that comprised 5 µL SYBR Green mix (Maxima SYBR Green/ROX qPCR Master Mix ×2, Thermo Fisher Scientific Inc., Waltham, MA, USA), 10 µM each of forward and reverse primers (2 µL) and 3 µL of template cDNA (equivalent amount of 3.75 ng mRNA). Amplifications were done in duplicates. Activation of DNA polymerase was achieved by incubating reactions for 10 min at 95 °C. The cDNA amplifications were performed by incubating the reactions for 40 cycles at 95 °C, for 15 s and 60 °C for 60 s. The Primer Express Software v3.0 (ABI 7900 HT, Applied Biosystems, Foster City, CA, USA) was used to design the primers used in the RT-PCR analysis. Primers used for the genes are shown in Table 3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene [58]. The relative mRNA quantifications were performed by comparing the genes of interest with the reference gene (GAPDH), and are expressed as arbitrary units, using the Real Time PCR Miner algorithm [59].

Table 3. Primers used in reverse transcription real-time PCR analysis.

Gene	Ensemble Accession No.	Primer Sequence	Start (bp)	Stop (bp)	Amplicon Size (bp)
TXNRD1	NM_214154.3	F: 5′-CCAAACCCAAGGCGAAGTTT-3′ R: 5′-GTGTAAGCACGGGACACGC-3′	1589 1654	1608 1636	65
HSPA4L	XM_021101609.1	F: 5′-GGAGGTTGCGGCGCAG-3′ R: 5′-CTAGGGCAGTGCGTTGGG-3′	35 93	50 76	58
ATP1B1	NM_001001542.1	F: 5′-CCATCTTCAATCCCCGCA-3′ R: 5′-GCTTTTCCGCGGGCC-3′	89 143	106 129	54

bp—base pairs.

4.6. GO Enrichment Analysis and KEGG Pathways

Enrichment analysis according to GO categories (MF, BF and CC) was performed, and the KEGG pathways of *TXNRD1*, *HSPA4L* and *ATP1B1* genes were analyzed, using the online tool g:Profiler (https://biit.cs.ut.ee/gprofiler/gost, accessed on 4 May 2022) [60]. The version of the g:Profiler was e105_eg52_p16_e84549f. Enrichment analysis was performed with the *sus scrofa* database, using the False Discovery Rate (FDR) of the Benjamini–Hochberg (BH) method for a significance threshold at 0.05.

4.7. Western Blotting Analysis

Sperm extracts were prepared from the fresh PF and FT sperm (50×10^6 spermatozoa/mL), according to a previously described protocol [21,56]. Western blotting analysis was performed on sperm extracts from boars of the GSF group (Boars no. 1–5) and those of the PSF group (Boars no. 6–10) to quantify protein expression in TXNRD1, HSPA4L and ATP1B1. Protein samples (30 µg/lane) were separated by SDS-PAGE [61] and moved to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) for immunodetection.

Electroblotting was performed according to a previously described method [62]. The procedure used for Western blotting analysis has been described in a previous study [21]. Following the blocking of the non-specific binding sites with 5% nonfat milk in Tris-buffered saline containing 0.05% v/v Tween 20 (TBST, MP Biomedicals LLC, Santa-Ana, CA, USA), blots were incubated overnight at 4 °C with the primary antibodies (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) against rabbit polyclonal TXNRD1 (TrxR1) antibody (PA5-34685; 1:500), rabbit polyclonal HSPA4L antibody (PA5-44098; 1:500) and mouse monoclonal ATP1B1 antibody (MA3-930; 1:500). Rabbit beta-actin antibody (β-actin, A2066; 1:500; Sigma Chemical Company, St. Louis, MO, USA) was used as the loading control. After incubation, the membranes were incubated for 2 h at room temperature with anti-rabbit IgG, horseradish peroxidase (HRP)-linked secondary antibody (111-035-003; 1:1000; Jackson ImmunoResearch, Baltimore Pike, PA, USA). Protein detection was performed by enhanced chemiluminescence (ServaLight CL EOS Substrate kit (Serva, Heidelberg, Germany)). Images were captured with the G:BOX iChemi XR imaging system (SynGene, Cambridge, UK), and protein bands were quantified using MultiAnalyst 1.1 software (Bio-Rad Laboratories, Hercules, CA, USA). The molecular weights of the protein were determined using the Molecular weight standard (Pre-stained Protein Ladder™, Unstained Protein Standards, Bio-Rad Laboratories, Hercules, CA, USA). Values are expressed as the total signal intensity inside the boundary of a band measured in pixel intensity units/mm^2—optical density (OD), using β-actin (Sigma Aldrich, Burlington, MA, USA) as the control to normalize the volume of the protein expression [63]. At least three replicates per boar were performed for the quantification of each analyzed gene in the fresh PF or FT sperm.

4.8. PPI Networks

We applied the Search Tool for the Retrieval of Interacting Genes database (STRING v11.5) available online (http://string-db.org, accessed on 12 August 2021) [64] to visualize the PPI networks for TXNRD1, HSPA4L and ATP1B1.

4.9. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

One dimensional SDS-PAGE and densitometry analyses were used to analyze the electrophoretic patterns of the proteins in the fresh PF and FT sperm, according to a previously described protocol [56], with some modifications. Briefly, protein samples (0.5 mg/mL) were precipitated on cold acetone (−20 °C), washed (14,000× g for 5 min), and the pellets were resuspended in a lysis buffer and heated for 5 min at 95 °C in a Thermo Block (TDB-120 thermostat (Biosan, Riga, Latvia). Electrophoresis was performed on 12% gels at 80 V for 15 min and then at 130 V for 1 h. Gels were stained with Coomassie Brilliant Blue R-250. The molecular masses of the protein bands were determined, PageRuler™ Prestained Protein Ladder standards (Thermo Fisher Scientific Inc.), while the band intensity was assessed

by densitometry analysis using MultiAnalyst 1.1 software (Bio-Rad Laboratories, Hercules, CA, USA). Analysis of the SDS-PAGE profiles included the total number of proteins and the difference in the electrophoretic profiles between the fresh PF sperm of the GSF and PSF groups or FT sperm of the GSF and PSF groups. Moreover, the protein fractions of the fresh PF sperm or FT sperm of each boar were categorized into five groups according to their molecular weights (<20 kDa, 21–40 kDa, 41–60 kDa, 61–80 kDa and >81 kDa) in order to assess the contribution of each fraction.

4.10. Statistical Analysis

Statistical analysis was performed with the Statistica software package, version 13.3 (TIBCO Software Inc., Palo Alto, CA, USA; StatSoft Polska, Kraków, Poland) We used the ANOVA assumption (Shapiro–Wilk test) was used to check the normality of the data distribution of the sperm parameters followed by the application of the Levene's test to examine for the homogeneity of variance. The general linear modeling (GLM) procedure was used for ANOVA analysis. A post-thaw TMOT cut-off threshold value of 30% was used for the classification of the freezability groups. Boars showing more than 30% (>30%) post-thaw TMOT were considered as having GSF, whereas those with TMOT lower than 30% (<30%) were classified as having PSF [21,65,66]. Assessment of the post-thaw semen quality showed that five boars (Boars no. 1–5) exhibited GSF, while five boars, (Boars no. 6–10) were considered having PSF. Descriptive variables of the sperm parameters are presented as the mean ± SEM, and the Student-t test was used for comparison between the GSF and PSF groups. The non-parametric Kruskal–Wallis ANOVA test was used to analyze VSL, VAP, VCL, ALH and BCF parameters, and the Mann–Whitney U test was used to compare the differences between the GSF and PSF groups.

The IBM SPSS Statistics software package (IBM Corp. released 2020. IBM SPSS Statistics for Windows, version 27.0, IBM Corp. Armonk, NY, USA) was used to analyze protein and gene expression. The Kruskal–Wallis 1-way ANOVA with a multiple comparison test was used to analyze the protein and gene expression levels among the groups: fresh PF–GSF, fresh PF–PSF, FT–GSF and FT–PSF. The gene and protein expression levels are presented as the mean ± SEM, and significant values were adjusted by the Bonferroni correction for the multiple comparison test. Values were considered significant at $p < 0.05$.

5. Conclusions

Taken together, the results of the present study confirm that cryopreservation has varying effects on the mRNA expression levels of *TXNRD1*, *HSPA4L* and *ATP1B1* in boar sperm, particularly those from the poor freezability ejaculates. The findings of the present study confirm that the relative expression levels of *ATP1B1* mRNA in the fresh PF sperm could provide useful information to discriminate between the good and poor freezability ejaculates. We suggest that *ATP1B1* mRNA expression in the fresh PF sperm is a promising sperm cryotolerance marker, while the expression analysis of TXNRD1 and HSPA4L protein in FT sperm provides useful information that may help to elucidate their biological significance in cryo-damage. These findings support future investigations on the functional relevance of the mRNA and protein expression levels of TXNRD1, HSPA4L or ATP1B1 in the cryotolerance of boar sperm.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23169320/s1.

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Olsztyn, 26.09.2022

Anna Katarzyna Mańkowska, M.Sc.

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Statement

I hereby declare that my participation in the publications, which are the basis of my doctoral thesis:

- Mańkowska A, Brym P, Paukszto Ł, Jastrzębski JP, Fraser L. 2020. Gene polymorphisms in boar spermatozoa and their associations with post-thaw semen quality. *International Journal of Molecular Sciences* 21(5), 1902.

Contributions: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing of original draft, editorial preparation and visualization.
The total contribution to the publication was 65 %.

- Mańkowska A, Brym P, Sobiech P, Fraser L. 2022. Promoter polymorphisms in *STK35* and *IFT27* genes and their associations with boar sperm freezability. *Theriogenology* 189, 199-208.

Contributions: methodology, validation, formal analysis, investigation, data curation, writing of original draft and editorial preparation.
The total contribution to the publication was 60 %.

- Mańkowska A, Gilun P, Zasiadczyk Ł, Sobiech P, Fraser L. 2022. Expression of *TXNRD1, HSPA4L* and *ATP1B1* genes associated with the freezability of boar sperm. *International Journal of Molecular Sciences* 23 (16), 9320.

Contributions: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing of original draft, editorial preparation and visualization.
The total contribution to the publication was 65 %.

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I hereby declare that my participation in the publications , which are the basis of the doctoral thesis of Anna Katarzyna Mańkowska, M.Sc.:

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Contributions: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing of original draft, editorial preparation, visualization, supervision, project administration and funding acquisition.
The total contribution to the publication was 15 %.

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Contributions: conceptualization, methodology, software, validation, formal analysis, investigation, writing of original draft, editorial preparation, visualization, supervision and funding acquisition.
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- Mańkowska A, Brym P, Sobiech P, Fraser L. 2022. Promoter polymorphisms in *STK35* and *IFT27* genes and their associations with boar sperm freezability. *Theriogenology* 189, 199-208.

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- Mańkowska A, Gilun P, Zasiadczyk Ł, Sobiech P, Fraser L. 2022. Expression of *TXNRD1, HSPA4L* and *ATP1B1* genes associated with the freezability of boar sperm. *International Journal of Molecular Sciences* 23 (16), 9320.

Contributions: formal analysis and editorial preparation.
The total contribution to the publication was 5 %.

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I hereby declare that my participation in the publications, which are the basis of the doctoral thesis of Anna Katarzyna Mańkowska, M.Sc.:

- Mańkowska A, Brym P, Paukszto Ł, Jastrzębski JP, Fraser L. 2020. Gene polymorphisms in boar spermatozoa and their associations with post-thaw semen quality. *International Journal of Molecular Sciences* 21(5), 1902.

Contributions: conceptualization, validation, formal analysis, data curation, editorial preparation and visualization.
The total contribution to the publication was 7.5 %.

- Mańkowska A, Brym P, Sobiech P, Fraser L. 2022. Promoter polymorphisms in *STK35* and *IFT27* genes and their associations with boar sperm freezability. *Theriogenology* 189, 199-208.

Contributions: methodology, software and validation.
The total contribution to the publication was 15 %.

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I hereby declare that my participation in the publication, which is the basis of the doctoral thesis of Anna Katarzyna Mańkowska, M.Sc.:

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Contributions: software, formal analysis and editorial preparation.
The total contribution to the publication was 7.5 %.

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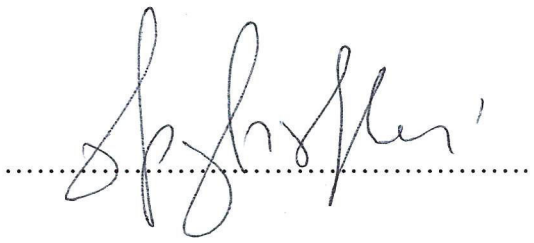
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Contributions: software, formal analysis and editorial preparation.
The total contribution to the publication was 5 %.



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I hereby declare that my participation in the publication, which is the basis of the doctoral thesis of Anna Katarzyna Mańkowska, M.Sc.:

- Mańkowska A, Gilun P, Zasiadczyk Ł, Sobiech P, Fraser L. 2022. Expression of *TXNRD1, HSPA4L* and *ATP1B1* genes associated with the freezability of boar sperm. *International Journal of Molecular Sciences* 23 (16), 9320.

Contributions: methodology, software and editorial preparation.
The total contribution to the publication was 5 %.



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Contributions: formal analysis and editorial preparation.
The total contribution to the publication was 5 %.

Łukasz Zasiadczyk