Genome-wide association studies for sperm traits in Assaf sheep breed

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A B S T R A C T
Sperm quality traits routinely collected by artificial insemination (AI) center for rams progeny test are related
with the capacity to produce sperm doses for AI and, in more or less grade, with males’ fertility. Low-quality ejacu-
lates are useless to perform AI sperm doses, which suppose high economic loses for the AI center. Moreover,
sperm quality traits have low heritability values which make traditional genetic selection little efficient to its
improvement. In this work, a genome-wide association study (GWAS) was conducted by using sperm quality traits
data and 50 K Affymetrix custom chip genotypes of 429 rams of Assaf breed from OVIGEN AI centre. Furthermore,
47 of these rams were also genotyped with the Illumina HD Ovine BeadChip, and therefore HD genotypes were
imputed for all rams with phenotype data. Previous to the GWAS, a linear regression model was fitted including
sperm traits as dependent variables; the flock of origin, date of sperm collection, and jump number as fixed ef-
fects; rams age at collection in months as covariate; and ram permanent effect as random. Pseudo-phenotypes
obtained from this model were used as input for GWAS. Associations at the chromosome-wise level (FDR 10%)
of 76 single-nucleotide polymorphisms (SNPs) in 4 chromosomes for ejaculate concentration (CON), 20 SNPs in
3 chromosomes for ejaculate volume (VOL), 32 SNPs in 1 chromosome for ejaculate number of spermatozoa
(SPZ), and 23 SNPs for spermatozoa mass motility (MOT) in 17 chromosomes were found. Only SNPs associated
with MOT overcame the genome-wise significance level. Some candidate genes for sperm traits variability were
SLC9C1 (OAR1), TGF (OAR2), and FUT10 (OAR26) for MOT; DOCK2, CPLANE1, SPEF2, and RAI14 (OAR16) for CON;
SCAPER and PSMA4 (OAR18) for VOL; and PARM1 and LOC101110593 (OAR6) for SPZ. SNPs associated with sperm
traits were not found to be correlated with milk production genetic variation; however, the high frequencies of
some SNPs with negative effect over sperm traits found in animals at the top milk yield estimated breeding values
(EBVs) ranking would allow to exert some selective pressure to improve rams sperm performances. Efforts and
frequencies of some of the SNPs detected over sperm quality traits make these variants good candidates to be
used in marker-assisted selection to improve sperm characteristics of Assaf rams and AI center efficiency to pro-
duce sperm doses.
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Implications
Finding out the genetic basis of sperm quality traits in the ovine spe-
cies is a very important issue since their moderate to low heritability
makes traditional selection methods little efficient to its improvement.
Sperm traits are highly related to artificial insemination centers effi-
ciency to perform rams progeny test in sheep dairy genetic programs.
The genome mutations associated with the variability of sperm traits
here detected could allow to perform a marker-assisted selection to
improve rams sperm characteristics, and therefore, their reproductive
ability and capacity to produce sperm doses.

Introduction
Artificial insemination (AI) is an essential reproductive tool in ge-
netic breeding programs of dairy ruminants. The use of this technique
enables progeny tests to predict estimated breeding values (EBVs)
of males, contributes to connect flocks, and is the best strategy to dissem-
nate the genetic improvement achieved by the genetic program using
elite rams. In sheep insemination centers, the fertilizing capacity of the
spermatozoa and the suitability of the semen for producing AI doses
are assessed using three parameters: the volume of the ejaculate

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(VOL), the concentration of spermatozoa (CON), and the spermatozoa mass motility (MOT) measured on a subjective scale. VOL and CON phenotypes are related to the effective number of doses that can be elaborated from one ejaculate, while sperm motility has been described as a good indicator of sperm fertilizing ability (Gadea, 2005; Broekhuysen et al., 2012). However, a direct relationship between these sperm parameters and ram’s fertility is not evident.

The moderate heritability estimates found for sperm characteristics in some sheep breeds (David et al., 2007; Pelayo et al., 2019) join to the fact that these traits are only collected in a very limited number of males (only rams from AI centers) makes conventional selection ineffective to improve them. Furthermore, the genetic correlation between dairy traits, milk yield and fat and protein contents, the main selection objective in most dairy sheep breeds, and sperm quality traits is unknown. If this correlation was negative, selection of elite rams for milk traits may lead to an impairment of ram’s spermatic quality.

Current genomic tools, such as medium and high-density genotyping single-nucleotide polymorphism (SNP) chips, could help to identify genomic regions associated with such low heritability traits, enabling to conduct marker assisted selection of sperm quality traits in livestock.

In last years, some works dealing with genome-wide association studies (GWAS) of sperm quality traits have been conducted in cattle (Suchocki and Szudy, 2014; Hering et al., 2014; Fonseca et al., 2018; Yin et al., 2019; Qin et al., 2017), pigs (Diniz et al., 2014; Marques et al., 2018), and goats (Wang et al., 2020). All of them were performed by using medium-density SNP genotyping arrays and a variable number of genotyped animals. Some putative candidate genes were associated with more or less degree with sperm mass motility, volume, concentration, and number of spermatozoa. In goats (Wang et al., 2020), a strong association of the DSCAML1 gene with sperm motility and density was found. However, to date, no studies dealing with genome-wide association of sperm traits in sheep have been conducted.

The main objective of this work is to identify SNPs and genomic regions associated with sperm quality traits by using genotypes from the 50 K Affymetrix custom chip and the Illumina HD Ovine BeadChip.

Material and methods

Data

Sperm quality traits were obtained from 27,635 ejaculates of 429 matured rams present in OVIGEN AI center, recorded between 2006 and 2018. Ejaculates were obtained after natural ejaculation in an artificial vagina. For a given ram, 1 or 2 successive ejaculates were collected over a 2–5 min period and evaluated immediately after collection. Three traits were assessed for each ejaculate: VOL (ml), which was measured using a graduated collection tube, CON (spermatozoa × 10^6/ml), which was determined using a standard spectrophotometer, and MOT, which was assessed for undiluted semen under a microscope. MOT was scored subjectively, based on wave motion on a continuous scale from 0 (no motion) to 5 (frequent rapid and vigorous waves) according to the original method described by Evans and WMC (1987). A fourth derived trait, the number of spermatozoa (SPZ) was computed as the product of VOL and CON and also included in the analyses. Data regarding these measurements are shown in Table 1.

Statistics

Phenotypes used in GWA studies were obtained from the sperm quality data described above. For that, in a first step, we performed a mixed linear regression analysis that allowed us to obtain a pseudo-phenotype for the GWAS of the second step. The four sperm traits, VOL, CON, SPZ, and MOT described above, evaluated in a total 27,635 ejaculates belonging to 429 matured rams, were considered as dependent variables in the analyses. A mixed linear regression analysis, including the ram flock of origin (51 levels), the date of sperm collection (1806 levels), and the ejaculate number (2 levels, first or second jump) as fixed effects; the age of the ram at sperm collection in months as a covariate; and the ram permanent environmental effect as random (429 levels), was fitted. Male’s individual estimates adjusted by factors included in the model were obtained and used as pseudo-phenotypes (inputs) in the GWAS. BLUPF90 suite (Misztal et al., 2002) was used to run the mixed linear regression analyses.

Once pseudo-phenotypes were obtained, a GWAS was carried out in a second step. From the 429 phenotyped rams, only 342 have 50 K SNP Affymetrix microarray genotypes (47,702 SNPs) in the current genotyped Assaf population. From these 342 males genotyped with the 50 K microarray, 47, were also genotyped with the HD Illumina OvineBeadChip (604,317 SNPs). Medium density 50 K and HD genotyping platforms have in common 43,511 SNPs. Imputation of 50 K to HD genotypes was conducted with BEAGLE4.0 software (Brown and Browning, 2007). Only genotypes with an imputation probability higher or equal to 95% were retained. After imputation and filtering by SNPs call rate (0.9), individual call rate (0.9), and MAF (0.001), a total of 540,411 SNPs distributed on the 26 ovine autosomes and X chromosome from 342 rams were included in subsequent analyses. GWAS was conducted with the mixed linear model based association analysis (MLMA) of the genome-wide complex trait analysis software (Yang et al., 2011) for the whole genome and including a genetic relationship matrix of origin to control for the random effects of genetic similarity and excluding the chromosome on which the candidate SNP is located (leaving-one-chromosome-out LOCO) applying the following model:

\[
y_{ij} = u + SNP_i + g_j + e_{ij}
\]

where \(y_{ij}\) is the pseudo-phenotype for the sperm trait analyzed for the genotyped animal; \(u\) is the overall mean; SNP is the effect of the \(i\) SNP (assumed as a covariate coded as 0, 1, or 2, respectively, to

Table 1

<table>
<thead>
<tr>
<th>Trait</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>27,635</td>
<td>1.23</td>
<td>0.57</td>
<td>0.1</td>
<td>8</td>
<td>0.46</td>
</tr>
<tr>
<td>Volume 1st ejaculate (ml)</td>
<td>21,043</td>
<td>1.31</td>
<td>0.57</td>
<td>0.1</td>
<td>5</td>
<td>0.43</td>
</tr>
<tr>
<td>Volume 2nd ejaculate (ml)</td>
<td>5,887</td>
<td>0.91</td>
<td>0.40</td>
<td>0.9</td>
<td>5</td>
<td>0.44</td>
</tr>
<tr>
<td>Concentration (&gt;10^6 spz.ml^-1)</td>
<td>26,414</td>
<td>3,978.64</td>
<td>1,328.49</td>
<td>120</td>
<td>9,701</td>
<td>0.33</td>
</tr>
<tr>
<td>Concentration 1st ejaculate (&gt;10^6 spz.ml^-1)</td>
<td>20,858</td>
<td>4,088.13</td>
<td>1,358.45</td>
<td>120</td>
<td>9,636</td>
<td>0.33</td>
</tr>
<tr>
<td>Concentration 2nd ejaculate (&gt;10^6 spz.ml^-1)</td>
<td>5,453</td>
<td>3,569.43</td>
<td>1,193.33</td>
<td>120</td>
<td>9,701</td>
<td>0.31</td>
</tr>
<tr>
<td>Number of spermatozoa (&gt;10^9)</td>
<td>26,411</td>
<td>5,191.88</td>
<td>3,165.30</td>
<td>109</td>
<td>37,968</td>
<td>0.61</td>
</tr>
<tr>
<td>Number of spermatozoa 1st ejaculate (&gt;10^9)</td>
<td>20,857</td>
<td>5,657.60</td>
<td>3,261.03</td>
<td>109</td>
<td>25,170</td>
<td>0.58</td>
</tr>
<tr>
<td>Number of spermatozoa 2nd ejaculate (&gt;10^9)</td>
<td>5,451</td>
<td>4,313.42</td>
<td>1,950.33</td>
<td>109</td>
<td>37,968</td>
<td>0.57</td>
</tr>
<tr>
<td>Motility</td>
<td>25,069</td>
<td>4.82</td>
<td>0.58</td>
<td>1</td>
<td>5</td>
<td>0.12</td>
</tr>
<tr>
<td>Motility 1st ejaculate</td>
<td>20,339</td>
<td>4.80</td>
<td>0.62</td>
<td>1</td>
<td>5</td>
<td>0.13</td>
</tr>
<tr>
<td>Motility 2nd ejaculate</td>
<td>5,360</td>
<td>4.89</td>
<td>0.42</td>
<td>1</td>
<td>5</td>
<td>0.09</td>
</tr>
</tbody>
</table>

N: number of records; Min: minimum value; Max: maximum value; Spz = spermatozoa number.
genotypes aa, Aa, and AA); \( g_i \) is the random additive genetic effect; and \( e_i \) is the residual error. Chromosome-wise significance association was assessed using a false discovery rate (FDR multi-test correction threshold of 10%). The choice of a threshold value of 10% is justified because this work is mainly exploratory and a first attempt to find variants associated with spermatic characters in sheep, so we decided to be more conservative. Visualization of the association results was performed in Manhattan plots and quantile-quantile plots using R software (R core Team, 2019).

Variant Effect Predictor from Ensembl (www.ensembl.org) was used to annotate significant SNPs detected at the genome and chromosome levels. The positional candidate genes were identified in the 250 Kb region on both sides of the significant SNPs according to the sheep genome assembly Oar_v3.1 and based on the Ensembl release 81. For functional protein interaction networks, the STRING software was used (Szklarczyk et al., 2019; https://string-db.org/).

To examine the frequencies of the SNPs associated with sperm traits detected in the GWAS analysis in an increased number of Assaf animals, we used the whole population currently genotyped in this breed, which consisted of 1503 (1077 males and 426 females) genotyped for the 50 K custom platform and imputed to the HD ovine BeadChip (the 1077 consisted of 1503 (1077 males and 426 females) genotyped for the 50 K scale), and total number of spermatozoa (SPZ; spermatozoa × 10⁶). Level of significance is represented by asterisks (***: \( P \leq 0.001 \); **: \( P \leq 0.01 \); *: \( P \leq 0.05 \)).

Results

The mixed-effects linear model of sperm traits

All the fixed effects included in the model, the ram flock of origin, the date of sperm collection, the ejaculate number, and the age of ram at the collection showed a significant effect on sperm traits. Fig. 1 shows the estimates and IC95% from the mixed-effects linear model for the date of sperm collection (expressed as month of collection, in Fig. 1), ejaculate number and age of ram in months for the four sperm traits considered. Ejaculates showed larger VOL and SPZ (although CON decrease, in part due to higher VOL) during the reproductive season (from July to next January). A decrease in VOL, CON, and SPZ from the first to the second ejaculate was observed but showing the second ejaculate higher motility than the first one. Regarding the age of ram, although significant, the magnitude of the effect was always very close to 0.

Genome-wide association study of sperm traits

After the QC of genotype data from 342 rams, a total of 540 411 SNPs distributed on the 26 autosomes and X ovine chromosome were included in subsequent analyses. Supplementary Table S1 shows genome-wide complex trait analysis results for significant SNPs at the chromosome and genome-wide level for the sperm traits studied. Genome-wide 10% FDR threshold for MOT was set on a \( P \)-value of \( 2.4 \times 10^{-5} \). Genome-wide 10% FDR thresholds corresponded to \( P \)-values ranging from \( 5.8 \times 10^{-6} \) to \( 1.2 \times 10^{-4} \) for MOT; from \( 2.3 \times 10^{-5} \) to \( 4.5 \times 10^{-5} \) for VOL; from \( 5.7 \times 10^{-6} \) to \( 4.8 \times 10^{-4} \) for CON; and from \( 2.3 \times 10^{-4} \) to \( 1.3 \times 10^{-4} \) for SPZ. Twenty-three SNPs distributed in 17 different chromosomes overcame the genome-wide significance level for MOT trait. For VOL, CON, and SPZ, significant associations were found only at the chromosome level in chromosomes: OAR6 (11 SNPs), OAR18 (6 SNPs), and OAR23 (3 SNPs) for VOL; OAR10 (3 SNPs), OAR13 (1 SNP), OAR16 (71 SNPs), and OAR26 (1 SNP) for CON; and OAR6 (32 SNPs) for SPZ.

MLMA and LOCO approaches yielded similar results for all traits analyzed, and only results for the LOCO approach are shown. Fig. 2 shows Manhattan and quantile-quantile plots of the GWAS analyses for the four sperm traits studied. Quantile-quantile was plotted to explore that actual distribution of \( P \)-values obtained followed the expected distribution and no other effects, such population structure, were missed. Supplementary Table S2 shows allele frequencies and effects of significant SNPs in the 342 rams used for the GWAS and in the current Assaf genotyped population (1503 animals).

For VOL, in most cases, the less frequent allele (A1) of the associated SNP showed a negative effect over the trait with allele substitution values (b) ranging between −0.07 and −0.15 and frequencies from 0.06 to 0.49. In some cases, the A1 allele had a low positive effect over VOL (0.07 to 0.09) with an intermediate allele frequencies (from 0.32 to 0.49) (Supplementary Table S2).

In general, the A1 alleles showed a negative effect over CON trait with b values from −407 to −179 and frequencies ranging between 0.05 and 0.5. However, also positive effects of the A1 alleles on CON were observed (from 181 to 242) at A1 frequencies between 0.25 and 0.50 (Supplementary Table S2).

In most cases, the A1 effect over SPZ trait was highly positive (from 426 and 760) or negative (from −451 to −700) with very similar frequencies (between 0.08 and 0.50) (Supplementary Table S2).

Interestingly, the A1 allele exerts a negative substitution effect with magnitudes ranging between −1.62 and −0.24 and frequencies between 0.03 and 0.34, for all significant SNPs detected in the GWA study over the MOT trait (Supplementary Table S2).

Supplementary Table S3 shows variant effect prediction results of significant SNPs associated with sperm traits. Table 2 shows putative causal genes located in the 250 Kb region on both sides of the significant SNPs for each trait, and the biological processes and molecular functions in which they are involved. Phenotypes, if exist, are also shown.

For VOL trait, associations at the chromosome level for 20 SNPs located in OAR6, OAR18, and OAR23 were found. Most variants were
located in introns from genes such as ARHGEF38 (Rho Guanine Nucleotide Exchange Factor 38) and PCDH7 (Protocadherin 7) in OAR6 and CABLES1 (Cdk5 Abl enzyme substrate 1) in OAR23. However, two missense variants (oar3_OAR18_30765484, and oar3_OAR18_30789799) with moderate effect (SIFT values 0.41 and 0.12, respectively) were found in the SCAPER (S-phase cyclin A-associated protein in the ER) gene in OAR18, with allele frequencies around 6% in both cases.

For CON, 76 SNPs significant associated with the trait were found in OAR10, OAR13, OAR16, and OAR26, being the most significant those found in OAR16 (71 SNPs). Also in this case, most variants were located in introns, upstream and downstream regions of genes such as DLEU7 (Deleted in lymphocytic leukemia 7) in OAR10; OSMR (Oncostatin M receptor), RANBP3L (RAN binding protein 3 like), NADK2 (NAD kinase 2, mitochondrial), SKP2 (S-phase kinase associated protein 2), CAPSL (Calcylphosine like), IL7R (Interleukin 7 receptor), SPEF2 (Sperm flagellar protein 2), and RAI14 (Retinoic acid induced 14) in OAR16; and SORBS2 (Sorbin and SH3 domain containing 2) in OAR26. Two synonymous variants with low effect, oar3_OAR16_1971417 and oar3_OAR16_36979589, were detected in DOCK2 (Dedicator of cytokinesis 2) and CPLANE1 (Ciliogenesis and planar polarity effector 1) genes of OAR16 with allele frequencies of 13 and 5.6%, respectively.
Table 2
Putative genes detected in the genome-wide association study for sperm traits in Assaf rams.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Chr</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Biological process, molecular function, phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>16</td>
<td>DOCG2</td>
<td>Dedicator of cytokinesis 2</td>
<td>Membrane raft polarization, immune response, chemotaxis, actin cytoskeleton organization</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>CYPANE1</td>
<td>Cilio genesis and planar polarity effector 1</td>
<td>Transmembrane protein, ubiquitous expression in testis (RPKM 2.7)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>RANBP7L</td>
<td>RAN binding protein 3 like</td>
<td>Intracellular transport, benign prostatic hyperplasia</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>NADK2</td>
<td>NAD kinase 2, mitochondrial</td>
<td>NAD metabolic process, regulation of systemic arterial blood pressure, calcium-mediated signaling</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>LMBRD2</td>
<td>LMBR1 domain containing 2</td>
<td>Integral component of membrane</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>CAPS1L</td>
<td>Calpocytesislike</td>
<td>Calcium ion binding, high expression in testis (RPKM 3.9)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>ILTR</td>
<td>Interleukin 7 receptor</td>
<td>Cell growth, homeostasis of number of cells, severe combined immunodeficiency</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>SPEF2</td>
<td>Sperm flagellar 2</td>
<td>Spermatogenesis, fertilization, sperm motility, immotile short-tail sperm</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>RA14</td>
<td>Retinoic acid induced 14</td>
<td>Nucleoplasm, mitochondrion, actin cytoskeleton, spermatogenesis</td>
</tr>
<tr>
<td>MOT</td>
<td>1</td>
<td>SLC3C1</td>
<td>Solute carrier family 9 member C1</td>
<td>Sperm motility, spermatogenesis, high expression in testis (RPKM 3.5)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>TSN</td>
<td>Translin</td>
<td>DNA-binding protein, very high expression in testis (RPKM 20.5)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>GRM8</td>
<td>Glutamate metabotropic receptor 8</td>
<td>Neuroactive ligand–receptor interaction, glutamategic synapse</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>PAQR3</td>
<td>Progestin and adipocytok receptor family member 3</td>
<td>Negative regulation of protein phosphorylation, most expressed in testis (RPKM 20.7)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>NCALD</td>
<td>Neurocalcin delta</td>
<td>Neuronal calcium sensor, regulation of systemic arterial blood pressure</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>DIP2C</td>
<td>Disco interacting protein 2 homolog C</td>
<td>Plasma uric acid levels, AMP binding</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>C3MIP</td>
<td>c-Maf inducing protein</td>
<td>T-cell signaling pathway, in utero embryonic development</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>CUL9</td>
<td>Cullin 9</td>
<td>Microtubule cytoskeleton organization, regulation of mitosis, most expressed in testis (RPKM 6.1)</td>
</tr>
<tr>
<td>VOL</td>
<td>6</td>
<td>ARHGEF38</td>
<td>Rho guanine nucleotide exchange factor 38</td>
<td>Activation of Rho protein signal transduction, most expressed in prostate (RPKM 3.4)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>TCTD2</td>
<td>Tigger transposable element derived 2</td>
<td>DNA binding</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>PCDH7</td>
<td>Protocadherin 7</td>
<td>Cell–cell recognition and adhesion, calcium ion binding</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>SCAPE</td>
<td>S-phase cyclin A associated protein in the ER</td>
<td>Nucleic acid binding, zinc ion binding, most expressed in testis (RPKM 4.5)</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>PSMA4</td>
<td>Proteasome subunit alpha 4</td>
<td>Core subalpha of the 20S proteasome, ubiquitin-dependent protein catabolic process</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>CABLES1</td>
<td>Cdk5 and Abl enzyme substrate 1</td>
<td>Protein binding, regulation of cell cycle, Body Mass Index</td>
</tr>
<tr>
<td>SPZ</td>
<td>6</td>
<td>CCSE1</td>
<td>Coiled-coil serine rich protein 1</td>
<td>DNA methylation, gastrointestinal microbiome,</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>KCNIP4</td>
<td>Potassium voltage-gated channel interacting protein 4</td>
<td>Regulation of potassium ion transmembrane transport</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>GBA3</td>
<td>Cytosolic beta-glucosidase</td>
<td>Beta-glucosidase catabolic process, protein stabilization</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>STIM2</td>
<td>Stromal interaction molecule 2</td>
<td>Cellular calcium ion homeostasis, activation of store-operated calcium channel activity</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>OCA2D1</td>
<td>OCA domain containing 1</td>
<td>Regulation of stem cell differentiation, protein binding, ovarian cancer</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>OHRH1</td>
<td>HOP homeobox</td>
<td>Chaperone-mediated protein assembly, negative regulation of cell differentiation, histone deacetylation</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>LOC1110593</td>
<td>RE1-silencing transcription factor-like</td>
<td>Negative regulation of cell proliferation, cellular response to electrical stimulus</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>PARNI</td>
<td>Prostate androgen-regulated mucin-like protein 1</td>
<td>Positive regulation of telomerase activity</td>
</tr>
</tbody>
</table>

Chr = chromosome; CON = sperm concentration; MOT = mass sperm motility; VOL = ejaculate volume; SPZ = number of spermatozoa.

For the trait SPZ, 32 associated SNPs were located in introns, upstream and downstream regions of genes at OAR6. Some of the target genes were CCSE1 (coiled-coil serine rich protein 1), KCNIP4 (potassium voltage-gated channel interacting protein 4), GBA3 (cytosolic beta-glucosidase), STIM2 (stromal interaction molecule 2), and PARN 1 (Prostate androgen-regulated mucin-like protein 1).

Finally, 23 SNPs associated at the genome-wide level with MOT were located dispersed across the whole genome at chromosomes 1, 2, 3, 4, 6, 7, 9, 10, 12, 13, 15, 17, 19, 20, 22, and 26. All SNPs were located in introns, 3’ UTR, splice and downstream regions of genes such as SLC9C1 (Solute carrier family 9 member C1) in OAR1; TSN (Translin) in OAR2; GRM8 (Glutamate metabotropic receptor 8) in OAR4; BMP2K (BMP2 inducible kinase) and PAQR3 (Progestin and adipocytok receptor family member 3) in OAR6; NCALD (Neurocalcin delta) in OAR8; DIP2C (Disco interacting protein 2 homolog C) in OAR13; C3MIP (c-Maf inducing protein) in OAR14; CUL9 (Cullin 9), CFB (Complement factor B) and C2 (Complement C2) in OAR20; and FUT10 (Fucosyltransferase 10) in OAR 26.

Discussion

Among the sperm traits collected routinely in AI centers, only mass sperm motility seems to be directly related with overall fertility (Colenbrander et al., 2003; Foote, 2003; Theau-Clément et al., 2011; David et al., 2015). However, sperm volume and concentration are traits with some interest regarding AI centers efficiency to produce semen doses. Heritability of ovine sperm traits ranges from very low to moderate. For MOT heritability in Lacaune and Assaf breeds takes values of 0.07 and 0.03, respectively (David et al., 2007; Pelayo et al., 2019). Moderate estimates of 0.19 and 0.12 have been found for CON and VOL, respectively, in Assaf breed. Higher estimates of 0.27 and 0.18 for CON and VOL, respectively, were found in Lacaune breed (David et al., 2007; Pelayo et al., 2019). Estimates of genetic correlation among these traits are variable across sheep breeds. In Manchega and Assaf dairy breeds, estimates of genetic correlations between VOL and CON were −0.49 and −0.25, respectively; between VOL and MOT, −0.29 and −0.33, respectively; and between CON and MOT, 0.32 and 0.20, respectively (Pelayo et al., 2019).

In addition, it has been described that intensive selection in milk production in cattle has been associated with an impairment of the fertilizing capacity (Berry et al., 2009). Therefore, the detection of genes associated with the phenotypes measured in AI centers is key to try to improve the reproducible properties of the sperm of AI rams. These features are more important in phenotypes such as MOT due to their low heritability and its relationships with the fertility of the seminal doses. In the study here conducted, some genomic regions have been associated with sperm quality traits, finding significant SNPs within or close to genes functionally related to reproductive traits in other species.

Ejaculate concentration

For CON, a strong signal has been detected at chromosome 16. The SNP oar3_OAR16_1971417 is a synonymous variant G > A located at
exon 32 of the DOCK2 gene. This gene encodes a large protein of 180 kDa involved in intracellular signaling networks and highly related to the immune response. DOCK2 deficiency has been related to placental abortion and prostate cancer. Furthermore, Liu et al. (2019) revealed that the methylation of DOCK2 in cattle was associated with a potential role in the regulation of male fertility, suggesting that sperm methylation patterns influence sperm fertility. Then, this SNP could be in linkage disequilibrium with other mutations in the promoter or regulatory regions of the gene that could affect CpG islands.

The SNP oar3_OAR16_38979589 (rs421824327) is a synonymous variant G > A at exon 11 of the CPI11 (cilogenesis and planar polarity effector 1) gene which is involved in several biological processes such as cilia assembly and protein localization to the ciliary transition zone. Mutations of this gene (also known as Jbts17) produce the Joubert syndrome, Meckel syndrome, and oral-facial-digital syndrome and abnormal spermatogenesis and also with infertility (Liu et al., 2020). The SNPs oar3_OAR18_29981728 A > C and oar3_OAR18_29985155 G > A are downstream gene variants of the PMSA4 (Prostate 20S subunit alpha 4) gene located at OAR18. PMSA4 is a component of the 20S core proteasome complex involved in the proteolytic degradation of most intracellular proteins. The 20S proteasome mediates ubiquitin-independent protein degradation, which is required in several pathways including spermatogenesis (20S-PA200 complex). Many proteins such as meiotic proteins, core histones, and unnecessary organelles are degraded during spermatogenesis. It has been demonstrated that the expression of PMSA8 (Proteasome 20S Subunit Alpha 8), which also be part of the 20S proteasome, in spermatocytes, is essential for mice male fertility (Zhang et al., 2019). However, female fertility does not require PMSA8, it is essential for mice male fertility (Zhang et al., 2019). Although there are no data about PMSA4 involvement in males' fertility, it should share some of the properties described for PMSA8, because it’s high expression in testis and role in the 20S proteasome.

The TET2 (Tet methylcytosine dioxygenase 2) gene is located 332 bp downstream the oar3_OAR6_19723487 at OAR6 (ARHGEF38). The protein encoded by this gene is a methylcytosine dioxygenase that catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine and plays a key role in active DNA demethylation. Expression levels of this gene during human spermatogenesis are pivotal for male fertility (Ni et al., 2016). Authors showed that levels of mRNA isoforms of the TET genes in spermatozoa associated with sperm parameters and were significantly reduced in subfertile men.

**Ejaculate number of spermatozoa**

For the ejaculate number of SPZ all significant associated SNPs were located at OAR6. The SNP oar3_OAR6_89628167 G > A is an intron variant located at PARM1 (Prostate androgen-regulated mucin-like protein 1) gene. Parm1 is a highly glycosylated, mucin-like type 1 transmembrane protein involved in the positive regulation of telomerase activity, in the survival of prostate cells (Cornet et al., 2003), and contributes to ovulation and/or luteal function by acting as a regulator of progesterone metabolism (Park et al., 2013). In cattle, a SNP (rs111027720) located in the PARM1 gene has been associated with the development of cleaved embryos to the blastocyst stage in “in vitro” fertilization procedures.
Allele frequencies of most significant single-nucleotide polymorphisms for sperm quality traits in the top and bottom 10% ranking for the milk yield estimated breeding values (EBV) of 1503 Assaf animals genotyped with the 50 K Affymetrix custom chip and imputed for the HD Illumina BeadChip.

<table>
<thead>
<tr>
<th>Marker ID</th>
<th>A1 &gt; A2</th>
<th>Gene</th>
<th>b</th>
<th>Trait</th>
<th>N</th>
<th>freq A1</th>
<th>freq A2</th>
<th>Diff</th>
</tr>
</thead>
<tbody>
<tr>
<td>oar3_OAR16_36979589</td>
<td>G &gt; A</td>
<td>CPANE1</td>
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<tr>
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<tr>
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<td>CON</td>
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<tr>
<td>oar3_OAR16_38445552</td>
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<tr>
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<tr>
<td>oar3_OAR18_29981728</td>
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<tr>
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<td>75</td>
<td>0.11</td>
<td>0.89</td>
<td>0.04</td>
</tr>
</tbody>
</table>

A1 = less frequent allele; b = allele substitution effect; N = number of records; Diff = frequency A1 in the EBV negative group – frequency A1 in the EBV-positive group. Trait = sperm trait (CON: sperm concentration; MOT: mass sperm motility; SPZ: number of spermatozoa; VOL: ejaculate volume).

Spermatogenesis

For MOT trait, SNPs associations were found dispersed across the whole genome. The SNP oar3_OAR1_1753597925 C > A at chromosome 1 is an intron 6 variant of the SLC9C1 (Solute carrier family 9 member Ct, also named Sperm-Specific Na(+)/H(+) Exchanger and Sperm-NHE) gene. SLC9C1 is a member of the sodium-hydrogen exchanger (NHE) family and is required for male fertility and sperm motility (Wang et al., 2003). Sperm-specific sodium/hydrogen exchanger is involved in intracellular pH regulation of spermatogenesis and required for sperm motility and fertility. Also has a role in sperm cell hyper activation, a critical step for sperm motility essential in the preparation of sperm for fertilization. SLC9C1 is an integral component of the plasma membrane as a channel transport of ions, glucose, other sugars, salts, organic acids, metal ions, and amino compounds. Fig. 3 (c) shows the functional protein association network of the SLC9C1 gene. The ADCY10 (Adenylyl cyclase type 10, Epidermis Secretory Sperm Binding Protein Li Ta) gene, a predicted functional partner, interacts and is co-expressed with SLC9C1. ADCY10 (also named Testicular Soluble Adenylyl Cyclase SAC) plays a critical role in mammalian spermatogenesis by producing the cAMP which regulates cAMP-responsive nuclear factors indispensable for sperm maturation in the epididymis. Genetic approaches have demonstrated that Adcy10 is necessary for male fertility and specifically for sperm motility and capacitation (Xie et al., 2006).

The variant oar3_OAR2_185913749 G > A is a 3'-UTR located at exon 6 of the TSN (Translin, also named Testis Brain-RNA Binding Protein) gene. The 3'-UTR region plays a crucial role in gene expression by influencing the localization, stability, export, and translation efficiency of an mRNA. The 3'-UTR can influence polyadenylation, translation efficiency, localization, and stability of the mRNA since it contains both binding sites for regulatory proteins as well as microRNAs. This gene is highly expressed in brain and testis and is involved in DNA damage repair and in mRNA transport (Cho et al., 2004; Wang et al., 2004). The protein Translin is involved in translational regulation during spermatogenesis, and binds to specific mRNAs in the testis, forming an RNP complex (Morales et al., 2002). Mice lacking the TB RBP gene (the TSN mouse orthologue) can sire offspring, but have reduced sperm production (Chennathukuzhi et al., 2003).

The SNP oar3_OAR26_27800594 C > A is an intron 2 variant of the FUT10 (Fucosyltransferase 10) gene. FUT10 encodes a fucosyltransferase protein with roles in protein folding, glycosylation, nervous system development, and fertilization. FUT10 is highly expressed in testis (6.67 TPM) and in cattle has been associated with the ejaculate volume (Qin et al., 2016).

Despite the moderate positive correlation existing between CON and MOT in Assaf sheep breed (0.20), any common gene or genomic region was found in the GWAS for both traits.

Relationship of candidate genes frequencies and estimated breeding values for milk yield

Relationship between sperm traits studied and milk yield was explored. For that, 1503 animals with genotypes mentioned above were ranked based on their EBVs for milk yield (personal communication, Assaf sheep breeding program). Then, top and bottom 10% animals were chosen, and allele frequencies for the most significant SNPs found in this work were compared between both, top and bottom animals. Table 3 shows allele frequencies of the most significant SNPs found for sperm quality traits in the 10% top and 10% bottom ranking of EBVs for milk yield in 1503 Assaf animals, and the difference between them in both groups (A1 EBVnegative-A1 EBVpositive).

In general for all traits, frequencies of the A1 alleles are very similar in both groups of animals. However, two SNPs for CON in the RA14 gene with negative effect over the trait and 2 SNPs for VOL in the PSMA4 gene with positive effect over the trait showed significant higher A1 frequencies in the group of animals with negative EBVs for milk yield. For the ejaculate number of SPZ the 3 SNPs located in the LOC101110593 gene, which exert a positive effect over the trait, have higher A1 frequencies in the group of animals with negative EBVs for milk yield.
frequencies in the top EBVs group of animals, but also that located in the PARM1 gene with negative effect over SPZ. Despite the small number of animals analyzed, it seems that these SNPs are not linked with genes involved in milk production. However, the high frequencies found for some SNPs with negative effect over sperm traits in the top milk yield EBVs group, would allow exerting some selection pressure over them to improve sperm characteristics in the high merit animals for milk production.

Most SNPs that were significantly associated with some sperm trait belong exclusively to the HD ovine Illumina BeadChip. This fact indicates that high-density SNP platforms are more suitable to detect genes or genomic regions related to animal characteristics, at least in this sheep breed. In general, sheep breeds show a low linkage disequilibrium than other livestock species, such as cattle and pigs, among markers from low density genotyping chips, as the 50 K Affymetrix platform. Some of the genes here detected in association with sperm quality traits could be used in marker assisted selection of rams to be used in AI centers for progeny test in dairy breeding programs.

In summary, the GWA studies conducted in this work have revealed the existence of genomic regions and some putative causal genes associated with sperm quality traits. Among them, SPEGZ for ejaculate concentration, SCAKER and PSM4H for ejaculate volume, PARM1 for ejaculate number of spermatozoa, and SLCC1 and FUT10 for sperm mass motility seem to be good candidate genes to improve sperm quality traits in sheep. More studies increasing the number of genotyped and phenotyped animals will be necessary to validate the magnitude of the associations here detected and to establish the possibility of using these genes to improve rams’ sperm traits.

Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal2020.100065.

Ethics approval

Not applicable.

Data and model availability statement

OVIGEN AI center is the owner of phenotypic data and genotypes from Assaf animals. None of the data were deposited in an official repository but available upon requests which must be accompanied by a description of what the data will be used for.

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Author contributions


Declaration of interest

None.

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References


