Animal xxx (2021) xxx

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

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

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ARTICLE INFO

Article history:
Received 6 May 2020
Received in revised form 20 August 2020
Accepted 21 August 2020
Available online xxxx

Keywords:
Association study
Pseudo-phenotypes
Rams
Single-nucleotide polymorphisms genotypes
Sperm parameters

ABSTRACT

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 ejaculates are unuseful 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 effects; 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-wide significance level. Some candidate genes for sperm traits variability were *SLC9C1* (OAR1), *TSN* (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 presure to improve rams sperm performances. Effects 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 produce sperm doses.

Implications

Finding out the genetic basis of sperm quality traits in the ovine specie 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 efficiency 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 genetic 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 disseminate 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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https://doi.org/10.1016/j.animal.2020.100065

(**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; Broekhuijse 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 Szyda, 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 Affiymetrix 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 $\times 10^6$/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 (Browning 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 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:

$$y_j = u + SNP_i + g_j + e_j$$

where y_j is the pseudo-phenotype for the sperm trait analyzed for the genotyped animal j; 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
Basic statistics of sperm traits data collected in the first and second ejaculates from Assaf rams.

Trait	N	Mean	SD	Min	Max	CV
Volume (ml)	27 635	1.23	0.57	0.1	8	0.46
Volume 1st ejaculate (ml)	21 643	1.31	0.57	0.1	5	0.43
Volume 2nd ejaculate (ml)	5 887	0.91	0.40	0.9	5	0.44
Concentration ($\times 10^6$ spz.ml^{-1})	26 414	3 978.64	1 328.49	120	9 701	0.33
Concentration 1st ejaculate ($\times 10^6$ spz.ml^{-1})	20 858	4 088.13	1 358.45	120	9 636	0.33
Concentration 2nd ejaculate ($\times 10^6$ spz.ml^{-1})	5 453	3 569.43	1 119.33	120	9 701	0.31
Number of spermatozoa ($\times 10^6$)	26 411	5 191.88	3 165.30	109	37 968	0.61
Number of spermatozoa 1st ejaculate ($\times 10^6$)	20 857	5 657.60	3 261.03	109	25 170	0.58
Number of spermatozoa 2nd ejaculate ($\times 10^6$)	5 451	3 433.42	1 950.33	109	37 968	0.57
Motility	25 699	4.82	0.58	1	5	0.12
Motility 1st ejaculate	20 339	4.80	0.62	1	5	0.13
Motility 2nd ejaculate	5 360	4.89	0.42	1	5	0.09

N: number of records; Min: minimum value; Max: maximum value; Spz = spermatozoa number.

genotypes aa, Aa, and AA); g_j is the random additive genetic effect; and e_j 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.emsembl.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 males included the 342 phenotyped rams used in the GWAS analysis).

Milk yield EBVs of the 10% top and 10% bottom ranking of these 1503 animals for the significant SNPs associated with sperm traits were studied in order to decipher the putative relationship between milk production efficiency and the frequencies of these variants.

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 2.4×10^{-5}. Chromosome-wide 10% FDR thresholds corresponded to *P*-values ranging from 5.8×10^{-6} to 1.2×10^{-4} for MOT; from 2.3×10^{-5} to 4.5×10^{-5} for VOL; from 5.7×10^{-6} to 4.8×10^{-4} for CON; and from 2.3×10^{-4} to 1.3×10^{-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 OAR 23 were found. Most variants were

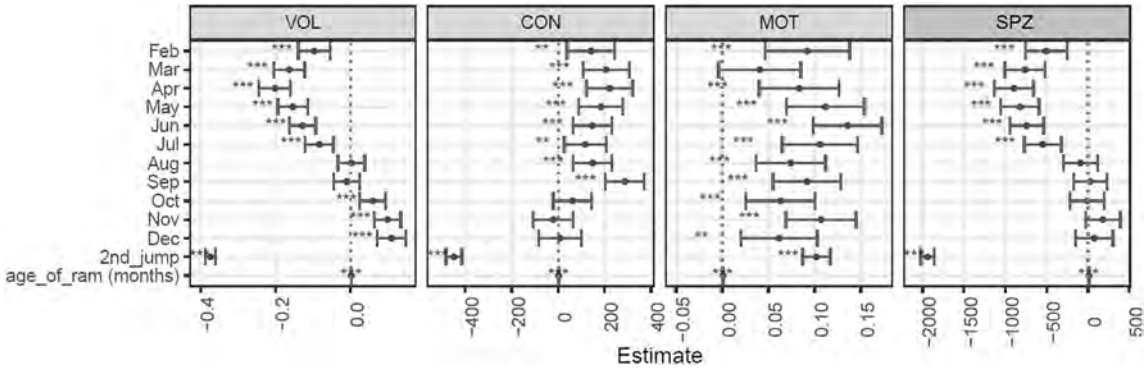

Fig. 1. Estimates (points) and IC95% (segments) from mixed-effects linear model for the date of sperm collection (expressed here as month of collection, Feb to Dec), ejaculate number (2nd ejaculate) and age of ram in months for the four sperm traits considered: sperm volume (VOL; ml, sperm concentration (CON; spermatozoa $\times 10^6$/ml, sperm motility (MOT; 0–5 scale), and total number of spermatozoa (SPZ; spermatozoa $\times 10^6$). Level of significance is represented by asterisks (***: $P \leq 0.001$; **: $P \leq 0.01$; *: $P \leq 0.05$).

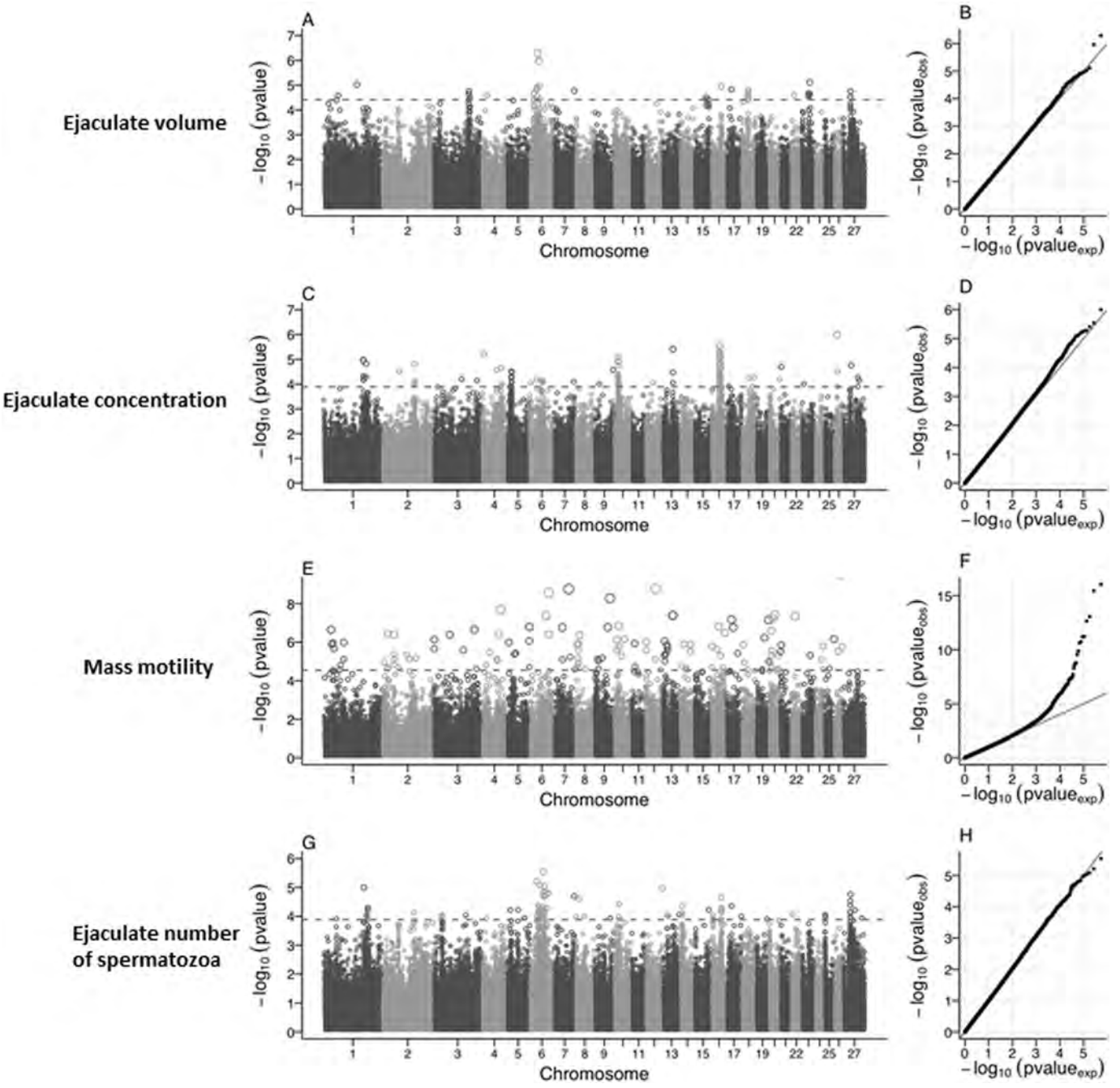

Fig. 2. Manhattan and Q-Q plots from genome-wide association study (GWAS) of sperm traits in Assaf sheep. Traits considered are ejaculate volume (ml; Figs. A and B); sperm concentration (sperm $\times 10^6$/ml; Figs. C and D); sperm motility (0–5 scale, Figs. E and F); and total number of spermatozoa (sperm $\times$ 10^6; Figs. G and H). Dashed line in Manhattan plots (A, C, E, and G) corresponds to average threshold value for a FDR of 10% evaluated at the chromosome level.

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* (*Calcyphosine 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 causal genes detected in the genome-wide association study for sperm traits in Assaf rams.

Trait	Chr	Gene symbol	Gene name	Biological process, molecular function, phenotype
CON	16	*DOCK2*	Dedicator of cytokinesis 2	Membrane raft polarization, immune response, chemotaxis, actin cytoskeleton organization
	16	*CPLANE1*	Ciliogenesis and planar polarity effector 1	Transmembrane protein, ubiquitous expression in testis (RPKM 2.7)
	16	*RANBP3L*	RAN binding protein 3 like	Intracellular transport, benign prostatic hyperplasia
	16	*NADK2*	NAD kinase 2, mitochondrial	NAD metabolic process, regulation of systemic arterial blood pressure, calcium-mediated signaling
	16	*LMBRD2*	LMBR1 domain containing 2	Integral component of membrane
	16	*CAPSL*	Calcyphosine like	Calcium ion binding, high expression in testis (RPKM 3.9)
	16	*IL7R*	Interleukin 7 receptor	Cell growth, homeostasis of number of cells, severe combined immunodeficiency
	16	*SPEF2*	Sperm flagellar 2	Spermatogenesis, fertilization, sperm motility, immotile short-tail sperm
	16	*RAI14*	Retinoic acid induced 14	Nucleoplasm, mitochondrion, actin cytoskeleton, spermatogenesis
MOT	1	*SLC9C1*	Solute carrier family 9 member C1	Sperm motility, spermatogenesis, high expression in testis (RPKM 3.5)
	2	*TSN*	Translin	DNA-binding protein, very high expression in testis (RPKM 26.5)
	4	*GRM8*	Glutamate metabotropic receptor 8	Neuroactive ligand–receptor interaction, glutamatergic synapse
	6	*PAQR3*	Progestin and adipoQ receptor family member 3	Negative regulation of protein phosphorylation, most expressed in testis (RPKM 20.7)
	6	*BMP2K*	BMP2 Inducible kinase	Skeletal development and patterning, protein phosphorylation
	9	*NCALD*	Neurocalcin delta	Neuronal calcium sensor, regulation of systemic arterial blood pressure
	13	*DIP2C*	Disco interacting protein 2 homolog C	Plasma uric acid levels, AMP binding
	14	*CMIP*	c-Maf inducing protein	T-cell signaling pathway, in utero embryonic development
	20	*CUL9*	Cullin 9	Microtubule cytoskeleton organization, regulation of mitosis, most expressed in testis (RPKM 6.1)
	20	*CFB*	Complement factor B	Complement activation, regulation of the immune reaction
	20	*C2*	Complement C2factor B	Complement activation, autoimmune diseases, response to nutrient
	26	*FUT10*	Fucosyltransferase 10	Fertilization, protein folding, wound healing
VOL	*6*	*ARHGEF38*	Rho guanine nucleotide exchange factor 38	Regulation of Rho protein signal transduction, most expressed in prostate (RPKM 3.4)
	6	*TIGD2*	Tigger transposable element derived 2	DNA binding
	6	*PCDH7*	Protocadherin 7	Cell–cell recognition and adhesion, calcium ion binding
	18	*SCAPER*	S-phase cyclin A associated protein in the ER	Nucleic acid binding, zinc ion binding, most expressed in testis (RPKM 4.5)
	18	*PSMA4*	Proteasome subunit alpha 4	Core alpha subunit of the 20S proteosome, ubiquitin-dependent protein catabolic process
	23	*CABLES1*	Cdk5 and Abl enzyme substrate 1	Protein binding, regulation of cell cycle, Body Mass Index
SPZ	6	*CCSER1*	Coiled-coil serine rich protein 1	DNA methylation, gastrointestinal microbiome,
	6	*KCNIP4*	Potassium voltage-gated channel interacting protein 4	Regulation of potassium ion transmembrane transport
	6	*GBA3*	Cytosolic beta-glucosidase	Beta-glucoside catabolic process, protein stabilization
	6	*STIM2*	stromal interaction molecule 2	Cellular calcium ion homeostasis, activation of store-operated calcium channel activity
	6	*OCIAD1*	OCIA domain containing 1	Regulation of stem cell differentiation, protein binding, ovarian cancer
	6	*HOPX*	HOP homeobox	Chaperone-mediated protein assembly, negative regulation of cell differentiation, histone deacetylation
	6	*LOC101110593*	RE1-silencing transcription factor-like	Negative regulation of cell proliferation, cellular response to electrical stimulus
	6	*PARM1*	Prostate androgen-regulated mucin-like protein 1	Positive regulation of telomerase activity

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 *CCSER1* (*coiled-coil serine rich protein 1*), *KCNIP4* (*potassium voltage-gated channel interacting protein 4*), *GBA3* (*cytosolic beta-glucosidase*), *STIM2* (*stromal interaction molecule 2*), and *PARM1* (*Prostate androgen-regulated mucin-like protein 1*).

Finally, 23 SNPs associated at the genome-wise 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 adipoQ receptor family member 3*) in OAR6; *NCALD* (*Neurocalcin delta*) in OAR9; *DIP2C* (*Disco interacting protein 2 homolog C*) in OAR13; *CMIP* (*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 reproductive 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

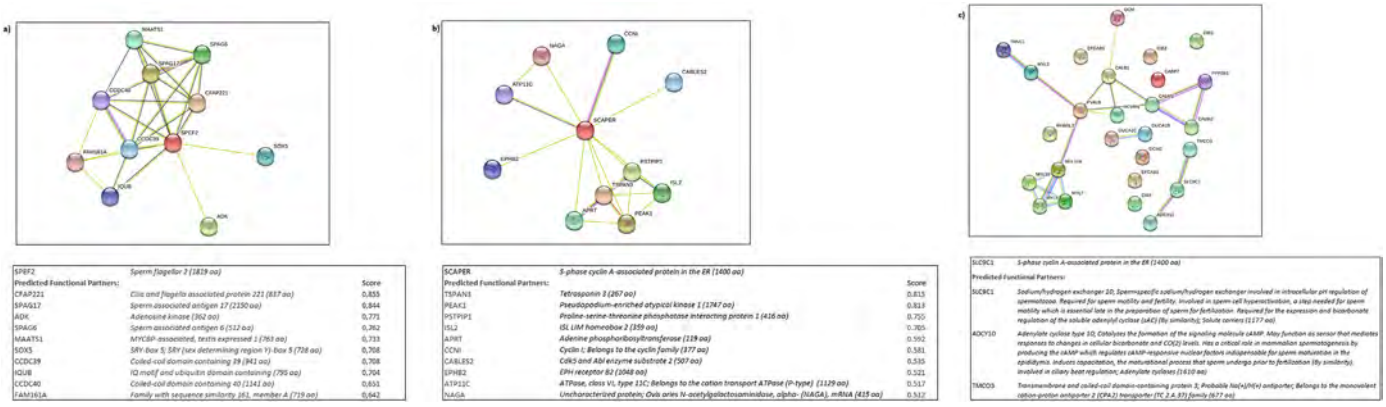

Fig. 3. Functional protein association networks of *SPEF2* (a) *SCAPER* (b) and *SLC9C1* (c) genes.

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 abruption 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 regulation regions of the gene that could affect CpG islands.

The SNP oar3_OAR16_36979589 (rs421824327) is a synonymous variant G > A located at exon 11 of the *CPLANE1* (*ciliogenesis and planar polarity effector 1*) gene which is involved in several biological processes such as cilium 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 cause ciliogenesis and ciliary trafficking defects resulting in a decreased of the *SHH (Sonic Hedgehog Signaling Molecule)* target gene expression (Toriyama et al., 2016). The sonic hedgehog signaling pathway is important in the murine epididymis for the development of sperm motility (Turner et al., 2006).

The SNPs oar3_OAR16_38356302 C > A, oar3_OAR16_38429385 A > G, oar3_OAR16_38445552 C > A, and oar3_OAR16_38480186 A > G are intron variants located at the *SPEF2* gene. *SPEF2* plays an important role in spermatogenesis and flagellar assembly (Sironen et al., 2011) and is expressed in all ciliated cells and required for cilia function (Sironen et al., 2010). Fig. 3 (a) shows the functional protein association network of the *SPEF2* gene. This network involves several genes related to sperm cells properties. In humans, variants of *SPEF2* gene are associated with many sperm anomalies such as oligozoospermia, short sperm flagellum, and abnormal spermatogenesis and also with infertility (Liu et al., 2020). Probably, these intron variants are not causal mutations affecting CON but should be in linkage disequilibrium with other mutations in coding regions of the *SPEF2* gene, not included in the genotyping platform.

Four SNPs, oar3_OAR16_39293892 G > A, oar3_OAR16_39321712 G > A, oar3_OAR16_39334360 G > A, and AX-124358876 (rs410614862) T > C, located at introns 1 and 2 of the *RAI14* (*Retinoic acid induced 14, also named NORPEG*) gene, were associated with the variability on CON. Rai14 is an actin binding protein which is expressed in the testis by both Sertoli and germ cells in the seminiferous epithelium. Rai14 regulates spermatid polarity and spermatid transport during spermiogenesis (Qian et al., 2013) and is also involved in the reorganization of actin filaments in Sertoli cells during the epithelial cycle, participating in conferring spermatid cell adhesion in the testis (Qian et al., 2013b).

Ejaculate volume

Two missense variants in OAR18, oar3_OAR18_30765484 G > A and oar3_OAR18_30789799 A > G located in exons 24 and 23 of the *SCAPER* gene, respectively, produce and aminoacid change of Threonine to Isoleucine and Phenylalanine to Leucine, respectively, that are predicted as tolerated changes with SIFT values of 0.41 and 0.12, respectively. The *SCAPER* (*S phase cyclin A-associated protein in the endoplasmic reticulum*) gene encodes a transmembrane transport protein which is highly expressed in testicle (ProteomicsDB, https://www.proteomicsdb.org/). In humans, *SCAPER* has been associated with retinitis pigmentosa (Tatour et al., 2017) and also with hypogonadism (a decreased functionality of the gonad) and abnormal testis morphology (Human Phenotype Ontology, https://www.genecards.org/cgi-bin/carddisp.pl?gene=SCAPER). Fig. 3 (b) shows the functional protein association network of the *SCAPER* gene.

The SNPs oar3_OAR18_29981728 A > C (rs415094151) and oar3_OAR18_29985155 G > A are downstream gene variants of the *PSMA4* (*Proteasome 20S subunit alpha 4*) gene located at OAR18. *PSMA4* 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 *PSMA8* (*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 *PSMA8* (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

Table 3
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 1 503 Assaf animals genotyped with the 50 K Affymetrix custom chip and imputed for the HD Illumina BeadChip.

					EBV milk yield negative			EBV milk yield positive			
Marker ID	A1 > A2	Gene	b	Trait	N	freq A1	freq A2	N	freq A1	freq A2	Diff
oar3_OAR16_36979589	G > A	*CPLANE1*	−407.02	CON	75	0.13	0.87	75	0.11	0.89	0.02
oar3_OAR16_1971417	G > A	*DOCK2*	−269.76	CON	75	0.21	0.79	75	0.13	0.87	0.08
oar3_OAR16_39293892	G > A	*RAI14*	−255.00	CON	75	0.34	0.66	75	0.29	0.71	0.05
oar3_OAR16_39321712	G > A	*RAI14*	−207.40	CON	75	0.37	0.63	75	0.29	0.71	0.07
oar3_OAR16_39334360	G > A	*RAI14*	−229.23	CON	75	0.41	0.59	75	0.26	0.74	0.15
AX-124358876	T > C	*RAI14*	−224.85	CON	75	0.46	0.54	75	0.30	0.70	0.16
oar3_OAR16_38429385	A > G	*SPEF2*	−290.53	CON	75	0.23	0.77	75	0.21	0.79	0.02
oar3_OAR16_38480186	A > G	*SPEF2*	−350.15	CON	75	0.25	0.75	75	0.21	0.79	0.05
oar3_OAR16_38356302	C > A	*SPEF2*	−206.52	CON	75	0.37	0.63	75	0.31	0.69	0.06
oar3_OAR16_38445552	C > A	*SPEF2*	−188.11	CON	75	0.42	0.58	75	0.47	0.53	−0.05
AX-169032210	A > G	*CFB/C2*	−0.63	MOT	75	0.06	0.94	75	0.06	0.94	0.00
oar3_OAR26_27800594	C > A	*FUT10*	−0.73	MOT	75	0.18	0.82	75	0.22	0.78	−0.04
oar3_OAR1_175397925	C > A	*SLC9C1*	−1.36	MOT	75	0.06	0.94	75	0.02	0.98	0.04
oar3_OAR2_185913749	G > A	*TSN*	−0.94	MOT	75	0.08	0.92	75	0.06	0.94	0.02
oar3_OAR6_72193906	A > G	*LOC101110593*	659.10	SPZ	75	0.13	0.87	75	0.16	0.84	−0.03
oar3_OAR6_72211624	A > G	*LOC101110593*	676.75	SPZ	75	0.06	0.94	75	0.13	0.87	−0.07
oar3_OAR6_72207209	G > A	*LOC101110593*	647.38	SPZ	75	0.11	0.89	75	0.16	0.84	−0.05
oar3_OAR6_89628167	G > A	*PARM1*	−538.34	SPZ	75	0.33	0.67	75	0.39	0.61	−0.06
oar3_OAR18_29981728	A > C	*PSMA4*	0.07	VOL	75	0.51	0.49	75	0.39	0.61	0.13
oar3_OAR18_29985155	G > A	*PSMA4*	0.08	VOL	75	0.56	0.44	75	0.43	0.57	0.13
oar3_OAR18_30789799	A > G	*SCAPER*	−0.15	VOL	75	0.10	0.90	75	0.04	0.96	0.06
oar3_OAR18_30765484	G > A	*SCAPER*	−0.15	VOL	75	0.11	0.89	75	0.07	0.93	0.04

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).

(Cochran et al., 2013a). The same authors found an SNP in the *PARM1* gene associated with some fertility traits such as heifers' conception rate, daughter pregnancy rate, and bull productive life in Holstein cattle (Cochran et al., 2013b). Three SNPs, oar3_OAR6_72193906 A > G, oar3_OAR6_72207209 G > A, and oar3_OAR6_72211624 A > G located at intron, upstream and downstream the *LOC101110593* (*RE1-silencing transcription factor-like*) gene, were positively associated with SPZ. *LOC101110593* is involved in whole organism decreased fertility and embryo development reduced rate (Thakore-Shah et al., 2015).

Spermatozoa mass motility

For MOT trait, SNPs associations were found dispersed across the whole genome. The SNP oar3_OAR1_175397925 C > A at chromosome 1 is an intron 6 variant of the *SLC9C1* (*Solute carrier family 9 member C1, 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 spermatozoa 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 amine compounds. Fig. 3 (c) shows the functional protein association network of the *SLC9C1* gene. The *ADCY10* (*Adenylate cyclase type 10, Epididymis Secretory Sperm Binding Protein Li 7a*) 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 SNP 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 *RAI14* 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 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 belongs exclusively to the HD ovine Illumina BeadCheap. 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, *SPEF2* for ejaculate concentration, *SCAPER* and *PSMA4* for ejaculate volume, *PARM1* for ejaculate number of spermatozoa, and *SLC9C1* 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.animal.2020.100065.

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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Malena Serrano: Conceptualization, formal analysis, investigation, resources, writing – original draft, writing – review & Editing. Manuel Ramón: Formal analysis, Writing – Original Draft. Jorge H Calvo: writing – review & editing, visualization. Mª Ángeles Jiménez: Data curation. Fernando Freire: Funding acquisition, resources. José Manuel Vázquez: Resources. Juan José Arranz: Writing – review & editing.

Declaration of interest

None.

Acknowledgements

We thanks ASSAF.E breeders association and OVIGEN to provide phenotype and genotype data of AI rams.

Financial support statement

This work was supported by the European Association for Innovation project 001958/G02 (FEADER), the contract INIA-GENOVIS Con19-043 and the National project RTA2013-00041-C02-01.

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