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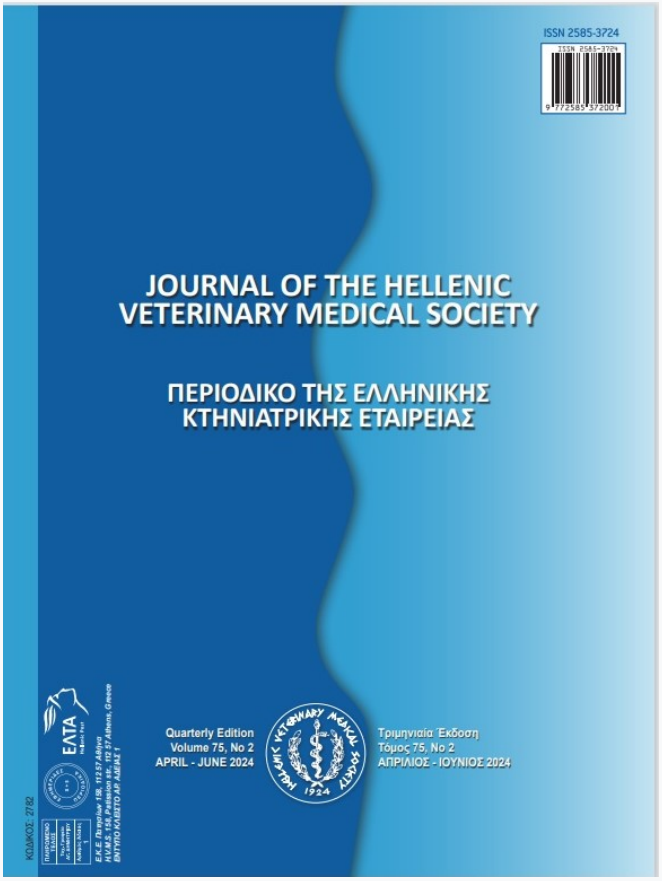

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Research article
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GDF9 gene c.260G>A mutation and sheep litter size: a meta-analysis

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ABSTRACT: Growth differentiation factor 9 (GDF9) has been identified as a major gene associated with sheep litter size. The most frequent single nucleotide polymorphism (SNP) associated with GDF9 is c.260G>A, altering protein sequence. Some studies found that this SNP did not affect litter size, while others found that it had an effect. This study examined the impact of this polymorphism on litter size through a meta-analysis. An analysis was conducted on six eligible published studies to examine the effects of c.260G>A polymorphism on litter size using four different genetic models: dominant (GG + GA versus AA), recessive (GG versus GA + AA), additive (GG versus AA) and co-dominant (GG + AA versus GA). The data were analyzed according to the I-squared value using fixed-effects and random-effects models. The c.260G>A polymorphism affected litter size significantly when analyzed as recessive (SMD = -0.24, 95 % CI [-0.41, -0.08]) and co-dominant (SMD = -0.36, 95 % CI [-0.64, -0.07]). In contrast, the c.260G>A polymorphism had no effect when used with dominant (SMD = 0.35, 95 % CI [-0.26, 0.96]) and additive (SMD = 0.18, 95 % CI [-0.78, 1.15]) genetic models. A statistical sensitivity analysis of pooled SMDs revealed no differences, indicating the overall results did not result from a single study. A meta-analysis shows that sheep litter size is related to genotype GA. This study supported the concept that GDF9 is a fundamental factor influencing sheep litter size.

Keywords: *GDF9* variation; meta-analysis; polymorphism; reproduction; sheep.

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INTRODUCTION

Sheep litter size is a crucial reproductive trait determined by genetics and the environment with a moderate heritability (0.16) (Alkass et al., 2021; Al-Thuwaini and Al-Hadi, 2022). Fecundity genes (*Fec*) control litter size genetically, and sheep with variants in these genes have more ovulation. Among them are three genes belonging to the superfamily of transforming growth factor (*TGF*) genes. These include bone morphogenetic protein 15 and receptor type 1B and growth differentiation factor 9 (*BMP15, BMPR-1B,* and *GDF9*) (Ajafar et al., 2022a; Ali et al., 2022). *GDF9* is derived from oocytes and contributes to sheep reproduction, including follicular development, oogenesis, and ovulation (Wang et al., 2021). *GDF9* also affects transmembrane receptor-protein serine/threonine kinases and is involved in ovulation cycles, gamete production, and gonadal development (Putra et al., 2022). The ovine *GDF9* gene resides on chromosome 5 with 2 exons and an intron that covers 1126 base pairs (bp). The long-mature peptide of this gene consists of 135 amino acids, while the prepropeptide has 453 amino acids (Muhaghegh Dolatabady and Habibizad, 2019). The encoded preproprotein encodes a factor essential for ovarian folliculogenesis. This factor stimulates the growth of granulosa cells and promotes primordial follicle development (Khodabakhshzadeh et al., 2016).

Genetic polymorphism of the *GDF9* gene has been shown to affect the fecundity traits of farm animals, with heterozygous genotypes resulting in increased ovulation rates and, consequently, increased prolificacy as compared to homozygous genotypes (Abdelgadir et al., 2021). Homozygous mutations of *GDF9* have been detected in infertile sheep, demonstrating its crucial role in this species (Khodabakhshzadeh et al., 2016). A study by Hanrahan et al. (2004) identified eight mutations (G1, G2, G3 to G8), five of which alter amino acid sequences. The *GDF9* gene strongly influences the fertility of Baluchi sheep (the largest native breed in Iran). The G1 mutation increased litter sizes in heterozygous animals relative to wild-type genotypes and homozygous carriers (Moradband et al., 2011). The SNP g.333G>A mutation significantly affects the litter size of Garut sheep (a local sheep in Indonesia). GG-genotyped ewes had significantly larger litter sizes than GA-genotyped ewes (Rahmawati et al., 2019). Al-Khuzai and Ahmed (2019) found notable litter size differences in dams with GA genotype in *GDF9* (exon 1).

There has been evidence that the *GDF9* gene polymorphism is related to litter size in several studies (Moradband et al., 2011; Al-Khuzai and Ahmed, 2019; Rahmawati et al., 2019; Hossain et al., 2020; Abdelgadir et al., 2021), whereas others have shown the association is insignificant (Talebi et al., 2018; Muhaghegh Dolatabady and Habibizad, 2019; Mohamed et al., 2020). The heterogeneity between the studies could be clarified by a meta-analysis (Medrado et al., 2021). By pooling data from numerous investigations, meta-analysis provides immense informational data to survey in a way that facilitates integrating the results obtained in various research studies (Bayraktar and Özdemir, 2022). Meta-analysis ensures that evaluations become more sensitive and accurate statistically (Mahmoudi et al., 2019; Medrado et al., 2021). A meta-analysis of the *GDF9* gene polymorphism was conducted on goats (Mahmoudi et al., 2019), but no studies were conducted on sheep. To better understand the correlation between litter size and *GDF9* polymorphism in different sheep breeds, a meta-analysis was conducted employing additive, dominant, codominant, and recessive genetic models.

MATERIALS AND METHODS

Methodology for identifying relevant studies

This study evaluated the *GDF9* gene influence on litter size in 1378 sheep from six breeds (Salsk, n = 500; Volgograd, n = 500; Mehraban, n = 115; Awassi, n = 49; Indigenous, n = 126; and Sudanese desert, n = 88). The 6 studies were from six countries Russia with a latitude of 61.5240° N, Iran with a latitude of 32.4279° N, Iraq with a latitude of 33° 00’ N, Bangladesh with a latitude of 24° 00’ N, and Sudan with latitude 15° 00 N. A PRISMA checklist was used in this meta-analysis to determine eligible studies. A comprehensive and precise investigation was performed to identify previous research articles associated with *GDF9* gene variation and litter size, which were published in various languages across multiple journals and databases. Studies published between 2002 and 2023 were used to conduct the research. Searches were conducted in PubMed, Springer, Wiley, Elsevier, Taylor & Francis, and Google Scholar. Studies were found using several keywords (litter size, polymorphism, *GDF9*, association, sheep).

Eligibility criteria for inclusion and exclusion

Inclusion criteria were the following: 1) describe the G1 SNP, (2) specify the genotype sample size, (3) examine the relationship between G1 SNPs and

litter size, (4) evaluate genotypes using least-squares means (LSM), and (5) provide a standard error for each genotype LSM. Furthermore, study exclusion criteria included: (1) abstracted studies, (2) insufficient data studies, (3) articles with duplicate data, and (4) reviews.

Data analysis

The selection of studies was analyzed in a meta-analysis based on inclusion-exclusion criteria. Data extracted include the first name of the author, year of publication, sheep breed and sample size, LSM, and standard error. A standard deviation calculation was required when analyzing the data, and this was done using standard errors of LSM and genotype sample sizes as follows:

SD = Standard error √ sample size for the genotype (Higgins et al., 2019).

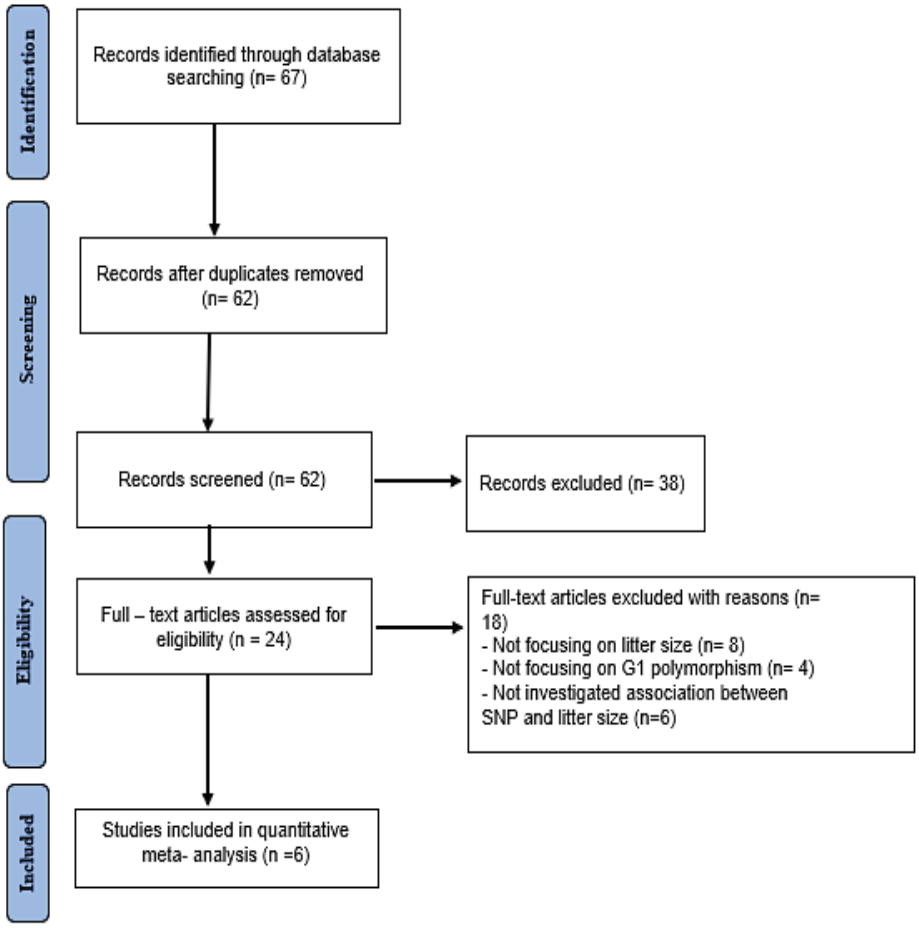
Analysis of statistical data

The data from various studies were analyzed using the recessive (GG vs. GA + AA), dominant (GG + GA vs. AA), additive (GG vs. AA), and co-dominant genetic models (GG + AA vs. GA) in version 5.0 of the review manager software package. The effect sizes were calculated using the standardized mean difference (SMD) approach, also called Cohen's d (Higgins et al., 2019). Based on Cohen's definitions, Cohen defined small, medium, and large effect sizes as 0-0.2, 0.21-0.5, and > 0.5. The heterogeneity of the effect sizes was assessed using Cochran's Q and I-squared (I^2). Data were fitted to the fixed-effects model that showed low heterogeneity ($I^2 < 50$ %), whereas data were fitted to the random-effects model that showed high heterogeneity ($I^2 > 50$ %). To assess the stability of the overall results, a sensitivity analysis was conducted by systematically eliminating one study at a time from the analysis. The final step of this process was to assess publication bias by analyzing Egger's test and funnel plots.

RESULTS

Study selection

A diagram of the PRISMA is shown in Figure 1. A database survey and a reference list screening result-



Figoure 1. PRISMA flow diagrams

ed in 67 references. The preliminary assessment discarded 5 papers as duplicates. Additionally, 38 reports were summaries and were therefore excluded. For the following reasons, 18 of the remaining 24 reports were discarded: (1) it was not examined whether the *GDF9* gene polymorphism impacted litter size, (2) there was insufficient data on genotype frequencies, standard deviation, and appropriate mutations; (3) litter size was not the focus of the research. Ultimately, 6 articles with 1378 sheep were selected for the meta-analysis. Each breed was studied independently in six papers; therefore, each breed was considered a separate study. A summary of the attributes of the chosen studies is presented in Table 1.

Heterogeneity assessment between studies

A comparison of heterogeneity results is shown in Table 2 using Cochran's Q test and I-squared statistic (I^2) measures. The Cochran's Q test showed that *P*-values under the co-dominant genetic model were 0.003. The I^2 for litter size was < 50% in genetic models (dominant and recessive), so a fixed-effect model was used. Other models considered also had estimated I^2 values greater than 50%. Accordingly, random-effect models were calculated to examine the effect of *GDF9* polymorphism on litter size under additive and co-dominant genetic models.

Polymorphism at c.260G>A and litter size: a meta-analysis

A meta-analysis of the association between SNPs and traits of interest is presented in Table 3 and Figures 3, 4, 5, and 6. The c.260G>A polymorphism was not associated with litter size under dominant (SMD

Table 1. A description of the characteristics of meta-analysis.

Study ID	Sheep breed	Country	Total sample	Genotype			LSM ± SE			Significant
				GG	GA	AA	GG	GA	AA	
Gorlov et al. 2018	Salsk	Russian	500	440	60	0	1.13 ± 0.09	1.80 ± 0.12	NE	Yes
Gorlov et al. 2018	Volgograd	Russian	500	80	420	0	1.22 ± 0.11	1.88 ± 0.17	NE	Yes
Tallebi et al. 2018	Mehraban	Iran	115	82	31	2	1.16 ± 0.18	1.17 ± 0.18	1.00 ± 0.18	No
Al-Khuzai and Ahmed, 2019	Awassi	Iraq	49	40	9	0	1.23 ± 0.07	1.33 ± 0.08	NE	Yes
Hossain et al. 2020	Indigenous	Bangladesh	126	65	57	4	1.59 ± 0.09	1.83 ± 0.10	2.00 ± 0.41	Yes
Abdelgadir et al. 2021	Sudanese desert	Sudan	88	53	30	5	1.24 ± 0.03	1.38 ± 0.04	1.04 ± 0.09	Yes

LSM ± SE, least square means ± Standard error.

Table 2. Research findings on meta-analysis heterogeneity.

Genetic model	Heterogeneity analysis			Examined model
	Q	*P* value	I^2 (%)	
Dominant (GG + GA vs. AA)	3.68	0.15	45.74	Fixed
Recessive (GG vs. GA + AA)	8.85	0.11	43.54	Fixed
Additive (GG vs. AA)	4.60	0.10	55.59	Random
Co-dominant (GG + AA vs. GA)	18.19	0.003	70.77	Random

Q, Cochran's test, I^2, I-squared test.

Table 3. A meta-analysis of litter size and the c.260G>A polymorphism.

Genetic model	No. breeds	SMD	95 % confidence interval		*P* value
			Lower limit	Upper limit	
Dominant (GG + GA vs. AA)	3	0.35	-0.26	0.96	0.26
Recessive (GG vs. GA + AA)	6	-0.24	-0.41	-0.08	0.004
Additive (GG vs. AA)	3	0.18	-0.78	1.15	0.71
Co-dominant (GG + AA vs. GA)	6	-0.36	-0.64	-0.07	0.01

SMD: standardized mean difference.

= 0.35, 95 % CI [-0.26, 0.96]) and additive (SMD = 0.18, 95 % CI [-0.78, 1.15]) models. However, the c.260G>A polymorphism showed a significant ($P \leq 0.05$) association with litter size under recessive and co-dominant genetic models (SMD = -0.24, 95 % CI [-0.41, -0.08]) and SMD = -0.36, 95 % CI [−0.07, 0.01]).

Sensitivity and publication bias analyses

A funnel plot was used to analyze publication bias in meta-analysis. Egger's regression test confirmed none of the genetic models had publication bias (P>0.05) (Table 4). An analysis of the stability of the pooled results included removing one study at a time from the meta-analysis. In the sensitivity analysis, pooled SMDs did not differ when individual studies were removed, indicating none of the single studies contributed to the overall outcomes. Figure 2 shows a funnel plot of genetic models.

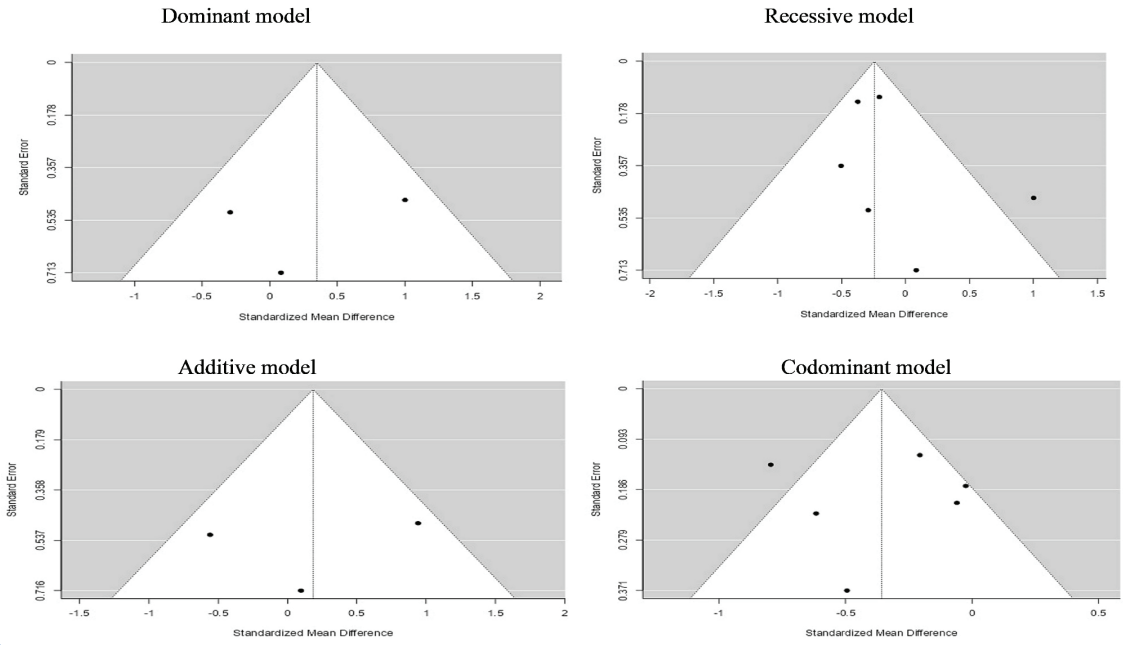


Figure 2. Funned plot showing the relationship between the observed effect size (standardized mea differences (SMD); solid circles) and its standart error for investigated different genetic models

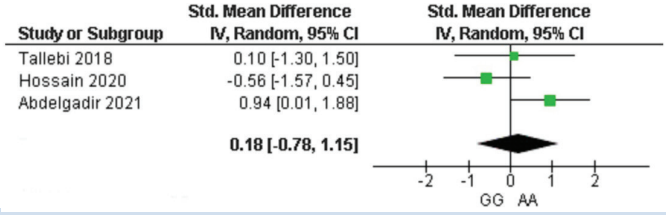


Figure 3. Forest plot for association between c.260G>A polymorphism and litter size applying the additive model. The thickness and length of the green rectangles indicate the weight and confidence interval of each study, respectively. The diamond placed at the bottom of the plot illustrates the summary effect

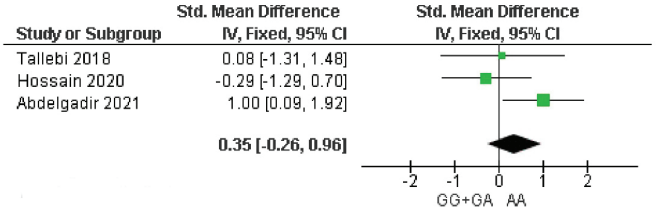


Figure 4. Forest plot for association between c.260G>A polymorphism and litter size applying the dominant model. The thickness and length of the green rectangles indicate the weight and confidence interval of each study, respectively. The diamond placed at the bottom of the plot illustrates the summary effect

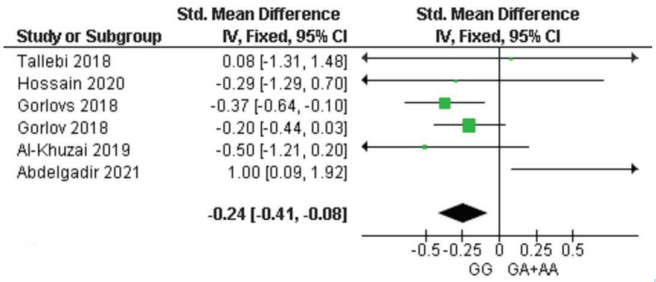


Figure 5. Forest plot for association between c.260G>A polymorphism and litter size applying the recessive model. The thickness and length of the green rectangles indicate the weight and confidence interval of each study, respectively. The diamond placed at the bottom of the plot illustrates the summary effect

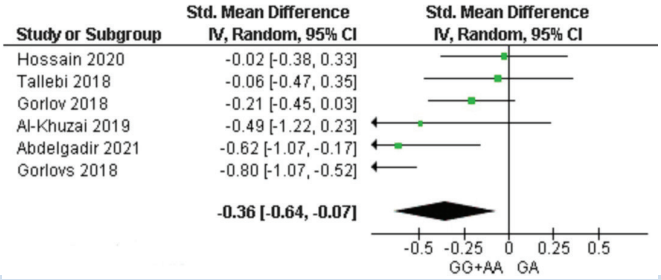


Figure 6. Forest plot for association between c.260G>A polymorphism and litter size applying the co-dominant model. The thickness and length of the green rectangles indicate the weight and confidence interval of each study, respectively. The diamond placed at the bottom of the plot illustrates the summary effect

Table 4. An investigation of publication bias using Egger's test.

Genetic model	Intercept	*P* value
Dominant (GG + GA vs. AA)	-0.79	0.42
Recessive (GG vs. GA + AA)	1.22	0.22
Additive (GG vs. AA)	-0.25	0.79
Co-dominant (GG + AA vs. GA)	-0.13	0.89

DISCUSSION

A significant goal of sheep breeding focuses on improving the productivity of sheep (Bayraktar and Shoshin, 2022a; Al-Jumaili et al., 2023). Domesticated sheep have been limited to breeding due to low fertility (Zhang et al., 2022). Relatively low efficiency is attributed to direct selection for fertility. This is due to two factors: trait heritability and sex-limitation of quality. Identifying of DNA markers responsible for these traits is a relevant and popular research topic (Gorlov et al., 2018; Ajafar et al., 2022b; Bayraktar and Shoshin, 2022b). Therefore, any knowledge of the function of these markers is useful to breeders for enhancing the ovulation rate and litter size in livestock (Kadhem and Al-Thuwaini, 2022; Al-Jaryan et al., 2023). The *GDF9* gene is essential for reproductive function in ewes (Wang et al., 2021). This gene has a dominant role in the granulosa, cumulus, and theca cells, and encourages folliculogenesis, oogenesis, and ovulation, which contribute to female fertility (Hossain et al., 2020). The oocyte *GDF9* controls many enzymes within the granulosa cells, which are responsible for ovulation, fertilization, and the successful completion of the reproductive process. It has been demonstrated that deletion of the *GDF9* gene blocks follicular development and results in infertility (Al-Mutar and Younis, 2020).

In sheep, variations in the *GDF9* gene influence litter size considerably. Breeders will benefit from this early selection and earn a substantial return on their investment (Wang et al., 2021). Numerous sheep breeds are studied for the polymorphism of the *GDF9* gene. Gorlov et al. (2018) examined the *GDF9* genetic variants and *their impact on Salsk and Volgograd sheep reproductive traits.* However, allelic substitutions (R87H/G1) did not influence Mehraban sheep litter sizes (Talebi et al., 2018). Although most stud-

ies have reported a significant association between polymorphisms in the *GDF9* and sheep litter size, reports still indicate that the association is insignificant. According to these contradictory findings, a meta-analysis could better understand the association between genetic variations and litter size. This could prove valuable in assessing the potential link between *GDF9* genetic variants and sheep litter size.

This study evaluated the *GDF9* gene influence on litter size in 1378 sheep cases of various breeds. According to a meta-analysis, genotypes of the *GDF9* gene significantly affected litter size ($P<0.01$) when using the codominant model. Results showed that the GA genotype had a significant effect on litter size. This study confirms the results of previous studies. Gorlov et al. (2018), Al-Khuzai and Ahmed (2019), and Abdelgadir et al. (2021) found that litter size is significantly higher in heterozygous GA genotypes. The ovulatory rate increases in heterozygous ewes, resulting in larger litter sizes; homozygous carriers of most mutations are infertile to varying degrees. A homozygous *GDF9* gene may prevent fat-tailed sheep breeds from functioning normally regarding reproductive hormonal pathways (Gorlov et al., 2018). In *GDF9*/G1, a missense substitution (R87H) is associated with comparatively higher prolificacy (Khodabakhshzadeh et al., 2016). This may explain why amino acid residue 87 in exon 1 is changed from arginine to histidine in G1, affecting the function of the GDF9 protein (Moradband et al., 2011). At the time of writing, there has been no meta-analysis on the relation between c.260G>A and sheep litter size. This meta-analysis produced substantial results by pooling numerous published studies. Sheep litter size and the *GDF9* gene were further investigated using different genetic models. In addition, to provide a precise answer concerning the c.260G>A polymorphism effect on litter size, the effect of individual studies was examined in sensitivity analyses.

The strengths of this meta-analysis are manifold: (i) it conducts an extensive review of meta-analysis studies in several languages, ensuring comprehensive coverage; (ii) it employs a sensitivity analysis to assess the consistency of results, systematically eliminating one study at a time; (iii) it thoroughly investigates the association between the *GDF9* polymorphism and sheep litter size by adopting four genetic models. The models included additive, dominant, codominant, and recessive models; and (iv) the meta-analysis utilized a large dataset containing 1378 records, which enabled more reliable findings. Nevertheless, the current meta-analysis study may contain some limitations: (i) the utilized genetic models showed moderate to high research heterogeneity; (ii) insufficient sample sizes were present in several studies; (iii) only genetic factors have been considered in determining sheep litter size when litter size is a complex trait. Accordingly, meta-analyses of future studies will also need to consider other factors, such as SNP-SNP, gene-gene interactions, correlative functional genes, and environmental effects.

CONCLUSION

A significant association was found between c.260G>A polymorphism and litter size in sheep under a codominant genetic model. Meta-analysis revealed that genotype GA increased sheep litter sizes. The *GDF9* gene can therefore be used in marker-assisted selection programs to increase litter size in sheep breeds with low reproductive performance. Introducing this gene by crossbreeding is another effective strategy for improving low-proliferous sheep breeds. The *GDF9* gene could thus be used in breeding programs, especially for sheep, to improve litter size. Nevertheless, more research, including gene knockout experiments, is required to confirm the benefits of *GDF9* gene variants in enhancing litter size.

CONFLICT OF INTEREST

None.

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