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Factors associated with canine skin extensibility in toy poodles

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ABSTRACT. To assess factors for canine skin extensibility, our study investigated associations between the dogs' skin extension index and the following factors, gender, age, neuter status, weight, coat color and six coat color related gene polymorphisms. Swab samples were collected from 69 toy poodles to extract DNA. The skin extension indices of the lower back and the neck were measured using the following formula: vertical height of the skin fold divided by body length multiplied by 100. The dogs' age, weight, gender, neuter status and coat color were also recorded, as well as polymorphisms of the following six selected coat color related genes, *Melanocortin 1 receptor*, *Tyrosinase-related protein 1*, *Melanophilin*, *Canine β-defensin-1*, *Major Facilitator Superfamily Domain Containing 12* and *Agouti-signaling protein* (*ASIP*). Univariable analysis showed there was a meaningful association between the lower back skin extension index and both gender and age ($P<0.001$ and $P=0.048$, respectively). Also, there was a possible association between the lower back skin extension index and *ASIP* Single nucleotide polymorphism (SNP) (R96C) ($P=0.078$). Linear model analysis showed there was a significant association between the lower back skin extension index and gender ($P<0.001$), and there was a tendency of the association between the lower back skin extension index and *ASIP* SNP (R96C) ($P=0.098$). In addition, there was an association between gender and age for the skin extension index. ($P=0.048$). Therefore, these results suggest that a greater risk of skin extensibility in toy poodle could be related to being female and the *ASIP* SNP (R96C), because these factors were associated with higher lower back skin extension index.

KEY WORDS: age, agouti-signaling protein, gender, skin extension index, toy poodle

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Canine skin disease is one of the most prevalent diseases in dogs. These diseases can impair dogs' health and wellbeing. Some of these diseases are related to collagen deficiency, and they can cause skin extensibility and fragile skin. For example, Ehlers-Danlos syndrome is a hereditary connective tissue disease that causes skin extension and fragility in various species including humans, dogs and cats. In dogs it has been reported that the disease is caused by collagen deficiency due to variants in the *collagen type V alpha 1 chain* gene [2]. Some veterinarians have noticed that fragile skin occurs more frequently in toy poodles with light coat color than in toy poodles with dark coat color. So, it is possible that skin extensibility and skin disease could be related to coat color, or to other factors such as gender, age, neuter status, body weight. A diagnostic criterium for skin fragility in dogs is the skin extension index (SEI) which measures the relative extendibility of the skin on the lower back or neck [19, 27]. A skin extension of 14.5% or more at the lower back or the neck is considered abnormal [27]. Abnormal SEI is a diagnostic criterion for Ehlers-Danlos syndrome and reflects a decline in dermal collagen [2].

Therefore, in this study we assessed potential factors that may influence canine skin extensibility by using univariable and linear model analysis to investigate the relationship between the SEI and dog gender, age, neuter status, body weight, and coat color. We hypothesized that coat color related gene polymorphisms are associated with skin extensibility and selected six candidate genes for analysis. These were the *Melanocortin 1 receptor* (*MC1R*) [20, 24] that controls which type of melanin is produced, either pheomelanin for red to yellow pigment or eumelanin for black to brown pigment, *Tyrosinase-related protein 1* (*TYRP1*) [10, 24] which encodes a melanosomal enzyme catalysing the oxidation of intermediates in the synthesis of eumelanin, *Melanophilin* (*MLPH*) [7, 26] which causes lightening of the coat color—i.e., from black to grey, brown to silver, or red to cream—as well as lightening the pigmentation of the nose, paw pads, and eye color, *Canine β-defensin-1* (*CBD103*) [4, 21] which is responsible for black coat color, *Major Facilitator Superfamily Domain Containing 12* (*MFSD12*) which causes cream to white color in many dog

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breeds [9] and *Agouti-signaling protein* (*ASIP*) which regulates the type, amount, and distribution pattern of the eumelanin (brown/black) and pheomelanin (yellow/red) [3, 12, 21, 23].

MATERIALS AND METHODS

Measurements

Sampling was performed on 69 toy poodles, aged from 0 to 15 years, in 2019 (Tables 1 and 2). Thirty samples (twenty-six non-neutered females, two neutered females and two non-neutered males) were collected at the petting zoo managed by "ZOOKISS Co., Ltd., Koshigaya, Japan" in TOBU ZOO (Saitama Prefecture), while the remaining 39 dogs (six non-neutered females, 10 neutered females, seven non-neutered males, 15 neutered males and one of unknown gender) were obtained from ordinary households in various regions (mainly Kanto area) in Japan. Since the gender and neuter status of one individual was unknown, the association analysis between the SEI and the phenotypic values was performed on only 68 dogs. The SEI was calculated by dividing the skin fold height on the lower back or neck by the length from the occipital crest to the radix caudae and multiplying by 100 (Fig. 1). When the SEI was more than 14.5%, it was considered to be an abnormal value [27]. The extension index measurements were recorded on the lower back of 68 dogs and on the neck of 39 dogs (Table 2). The coat color of each dog was categorized as either black, gray, red, cream, or white by visual judgement from the pedigree description of the sampled animal or by a photograph for sampled animals which no pedigree description was available. The gender, age, neuter status, and body weight of each dog was also recorded (Tables 1 and 2). This experiment has been approved by the institutional animal care and use committee of Meiji University (approval ID#: MUIACUC2020-130).

Table 1. Details of qualitative variables

Records of 69 dogs		N	%
Breed	Toy poodle	69	100
Gender	Male	24	34.8
	Female	44	63.8
	Unknown	1	1.4
Neuter status	Neutered (Male)	15	21.7
	Intact (Male)	9	13.0
	Neutered (Female)	12	17.4
	Intact (Female)	32	46.4
	Unknown	1	1.5
Coat color	Black	12	17.4
	Gray	2	2.9
	Red	37	53.6
	Cream	15	21.7
	White	3	4.4

Table 2. Details of quantitative variables

Records of 68 dogs	N	Average (range)
Age (years)	68	7.07 (0–15)
Body weight (kg)	68	3.57 (1.25–7)
Length of Occipital Crest-Radix caudae (cm)	68	34.67 (22–46)
Extension of lower back skin (cm)	68	2.52 (0.5–4.5)
Extension of neck skin (cm)	38	3.12 (1–5)
Lower back skin extension index (%)	68	7.36 (1.72–13.04)
Neck skin extension index (%)	38	8.64 (2.4–17.24)

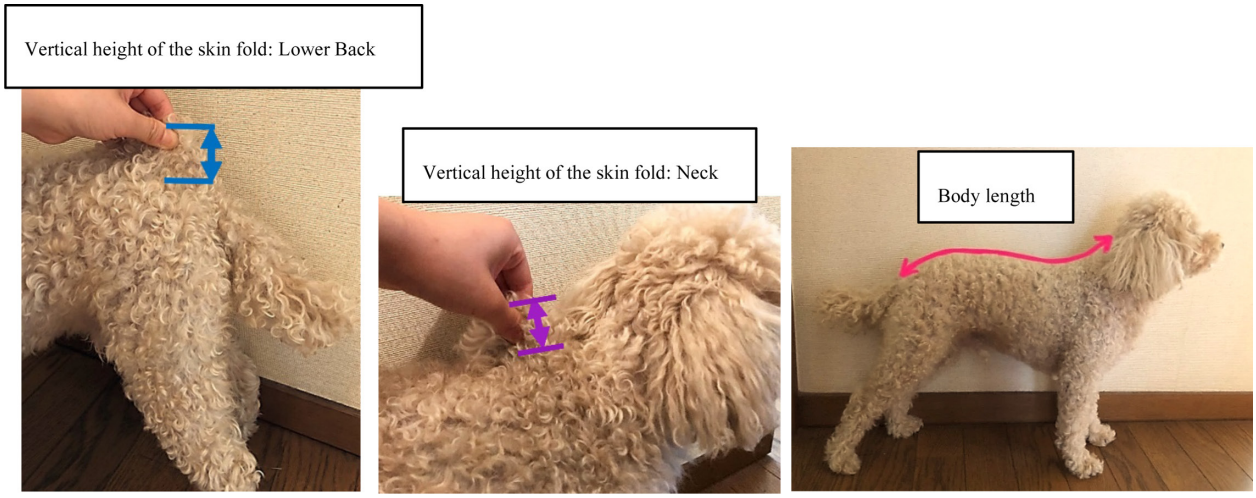

Fig. 1. Method for measuring the skin extension index (SEI).

DNA extraction and polymorphism analysis

After collecting canine oral mucosal epithelial cells using the swab method, DNA was extracted using a Saliva DNA Isolation Kit (NORGEN, Ontario, Canada). Total DNA was amplified with KOD FX Neo (TOYOBO, Osaka, Japan). Details of each primer are shown in Table 3. The PCR products were purified using either the ethanol precipitation method or the bead precipitation method (AMPure XP, Beckman Coulter, Brea, CA, USA). The purified PCR products were sequenced using a BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA), and then nucleotide sequencing was performed by a 3130xl genetic analyzer (Applied Biosystems). MEGA 6.06 [25] was used to align the sequence data.

Statistical analysis

Statistical analysis was performed using the free statistical software EZR version 1.54 [11]. In this study, the SEI was used as the response variable, while age, gender, neuter status, body weight and coat color were used as explanatory variables. The lower back and the neck skin extension indices, age, gender, and body weight all showed normal distributions, so a parametric test was performed. In the univariable analysis of the SEI, a *t*-test was used to assess the relationship with the qualitative variables (neuter status and coat color), and a Pearson correlation coefficient was used to assess the relationship with the quantitative variables (age, gender and body weight). Linear model analysis was performed by R version 4.0.3 [22]. Stepwise linear regression analysis was performed with backward elimination to determine factors associated with lower back SEI. Also, possible two-way interactions between explanatory variables were examined. A two-way interaction means that the effect of one variable on an outcome variable depends on the value of another variable [16].

RESULTS

Descriptive statistics data

The qualitative and quantitative variables of the sampled toy poodles are shown in Tables 1 and 2, respectively. There were samples from 24 male dogs, 44 females, and one individual of unknown gender. The average age of the dogs was 7.07 years, ranging from 0 to 15 years of age. The mean SEI of the 68 dogs, excluding the dog of unknown gender, was 7.36% on the lower back and 8.64% on the neck. Only two dogs had SEI values exceeding 14.5%, which is considered to be outside the normal range; both of these values were for neck skin extension measurements.

Association between SEI and phenotypes

The skin extension indices of the lower back and the neck were used as the response variables for association analysis with the explanatory variables of age, gender, neuter status, body condition and coat color. In the association analysis between each qualitative variable and each SEI, a *t*-test was performed because the data were normally distributed, the two groups were

Table 3. Primers used for amplification and sequencing

Gene	Primer Name	Target	Direction	Sequence	Reference	Reference for primer design
MC1R	MC1R_Ex1_F1	Ex1	F	CTGCAACTCCATCATTGACC	[24]	[24]
MC1R	MC1R_Ex1_R1	Ex1	R	CTGCCCAGCACCCTGGCCTC	[24]	[24]
TYRP1	TYRP1_Ex1_F1-1	Ex1	F	GAAGCATCTTCTTGTTCCTG	[10, 24]	This study
TYRP1	TYRP1_Ex1_R1-1	Ex1	R	TCTGGAGTCTGCTATCACTG	[10, 24]	This study
TYRP1	TYRP1_Ex1_F1-2	Ex1	F	ATCAGACCTGGGCTCAATTC	[10, 24]	This study
TYRP1	TYRP1_Ex1_R1-2	Ex1	R	GGTCTGGAGTCTGCTATCAC	[10, 24]	This study
TYRP1	TYRP1_EX4_F1	Ex4	F	CTCACTGTAATGCCCCTAAG	[10, 24]	This study
TYRP1	TYRP1_EX4_R1	Ex4	R	CTCGAGCAGAGAATTGTGAC	[10, 24]	This study
TYRP1	TYRP1_Ex4_F1-1	Ex4	F	GCCAATTAGGAGAAATCCAG	[10, 24]	This study
TYRP1	TYRP1_Ex4_R1-1	Ex4	R	TACCTTCCACTGTGTTTCGG	[10, 24]	This study
TYRP1	TYRP1_Ex4_F1-2	Ex4	F	AGCTGGAAATGTGGCTAGAC	[10, 24]	This study
TYRP1	TYRP1_Ex4_R1-2	Ex4	R	CTTACCTTCCACTGTGTTTC	[10, 24]	This study
MLPH	MLPH_Ex1_Fa	Ex1	F	TTCCTTCCCCTGTAGGACCG	[7, 26]	This study
MLPH	MLPH_Ex1_Ra	Ex1	R	ACCGTGGACCCTCCTTATGC	[7, 26]	This study
MLPH	MLPH_Ex7_Fa	Ex7	F	TCTCCTTCCACGACTTGGAC	[7, 26]	This study
MLPH	MLPH_Ex7_Ra	Ex7	R	GCTCAGGTTATCCTGGGAAG	[7, 26]	This study
MLPH	MLPH_Ex7_Rb	Ex7	R	CGAAGCCTGATATGGCCATC	[7, 26]	This study
CBD103	f-defensinS54m-23	Ex2	F	GTATGTCTTCATCCCTGTGAGGT	[4, 21]	[21]
CBD103	r-defensinS54m-23	Ex2	R	CTTCCAGGAGGCATTTTCACACT	[4, 21]	[21]
MFSD12	MFSD_Ex1_F1	Ex1	F	CTTCCTCAATGACCTGTGCG	[9]	[9]
MFSD12	MFSD_Ex1_R1	Ex1	R	CCGCGACTTACCAACCAG	[9]	[9]
ASIP	Agouti-exon4F-1–23	Ex4	F	CGAGACAGACGTGAGGACAGGTG	[3, 12, 21, 23]	[21]
ASIP	Agouti-exon4R–283L	Ex4	R	TAGATAATCAGCCAACCCCCTGGA	[3, 12, 21, 23]	[21]

independent, and the *F*-test showed homoscedasticity. A significant association was found between the lower back SEI and gender ($P<0.001$) (Fig. 2), but not with either neuter status or coat color ($P=0.197$ and $P=0.153$, respectively). There were no associations between neck SEI and any qualitative variable (Supplementary Fig. 1). For the association analyses between the skin extension indices and the quantitative variables, a Pearson correlation coefficient was used because both groups followed a normal distribution. There was a significant association between the lower back SEI and age ($P=0.048$) (Fig. 3), but not between SEI and body weight ($P=0.343$). Also, there was no significant association between neck SEI and either age or body weight ($P>0.1$) (Supplementary Fig. 2).

Association between SEI and coat color related gene polymorphisms

None of the 69 individuals had mutations in any of the three *MLPH* gene polymorphisms or in the two *TYRP1* gene polymorphisms, so these polymorphisms were excluded from the analysis (Table 4). Therefore, association analysis was performed between the two skin extension indices and seven gene polymorphisms in the five remaining candidate genes. There were no associations between the lower back SEI and any polymorphisms of the *MC1R*, *TYRP1*, *MFSD12* or *CBD103* genes. Also, while there were no significant associations between the lower back SEI and the A82S and R83H polymorphisms of the *ASIP* gene ($P=0.174$ and $P=0.174$, respectively), there was a suggestive association with the lower back SEI for the R96C polymorphism ($P=0.078$) (Fig. 4). However, none of the gene polymorphisms in any of the four genes were significantly associated with the neck SEI (Supplementary Fig. 3).

Linear model analysis

Based on the associations between the lower back SEI and the age, gender and *ASIP* SNP (R96C) factors shown in Figs. 2, 3 and 4, respectively, a linear model was constructed using the three factors and their three two-way interactions for lower back SEI (Table 5). However, the associations between SEI and two of the two-way interactions were not significant [*ASIP* SNP (R96C) and Age: $P=0.909$, and *ASIP* SNP (R96C) and Gender (Male): $P=0.909$]. Next, stepwise linear regression analysis with backward elimination

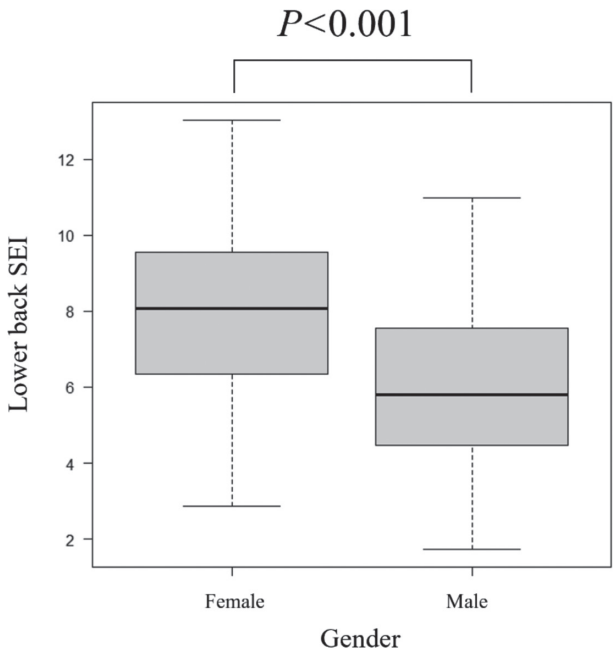

Fig. 2. Comparison of the lower back skin extension index (SEI) between female and male dogs.

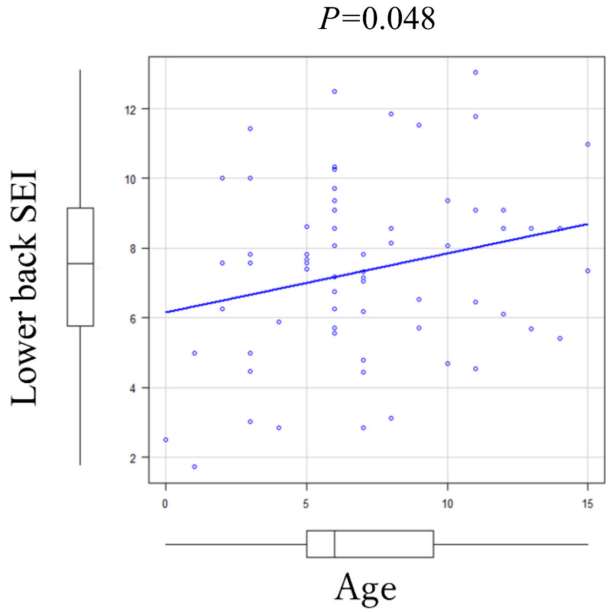

Fig. 3. Association between dog age and lower back skin extension index (SEI).

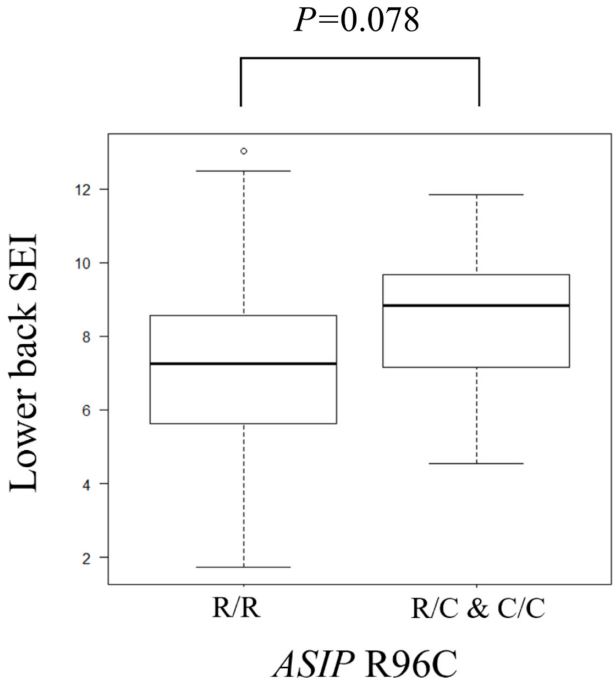

Fig. 4. Comparison of the lower back skin extension index (SEI) for different *ASIP* R96C polymorphisms.

was performed, excluding associations with the lowest *P*-value at each step. After excluding two associations, *ASIP* SNP (R96C), age, gender and the association between age and gender remained in the final model (Table 6). Note that age remained in the model because the two-way interaction between age and gender was significant (P=0.048). The analysis showed a significant association between the lower back SEI and gender (P<0.001), as well as a suggestive association between the lower back SEI and *ASIP* SNP (R96C) (P=0.098). In addition, an association was observed between [age and gender] (P=0.048).

Table 4. Genotype of each gene polymorphism for association analysis

Gene	Polymorphism	Sample (N)	Genotype		
MC1R	c.916C >T	69	C/C	C/T	T/T
			6	8	55
TYRP1	c.121T >A	69	T/T	T/A	A/A
			60	9	0
TYRP1	c.991C >T	69	C/C	C/T	T/T
			69	0	0
TYRP1	c.1033_1035del	69	WT	Deletion	-
			69	0	
MLPH	c.-22G >A	69	G/G	G/A	A/A
			69	0	0
MLPH	c.667_668insC	69	WT	Insertion	-
			69	0	
MLPH	c.705G >C	69	G/G	G/C	C/C
			69	0	0
CBD103	CBD103ΔG23	69	WT	ΔG23	-
			32	37	
MFSD12	c.154C >T	69	C/C	C/T	T/T
			44	16	9
ASIP	A82S	69	A/A	A/S	S/S
			59	9	1
ASIP	R83H	69	R/R	R/H	H/H
			59	9	1
ASIP	R96C	69	R/R	R/C	C/C
			57	11	1

Table 5. Associations between lower back skin extension index (SEI) and *ASIP* SNP (R96C), gender, age, and two-way interactions between these variables, based on linear model analysis

	Lower back SEI		
	Estimate	Std. Error	*P*-value
Intercept	7.561	0.791	<0.001
ASIP gene R96C (R/C & C/C)	1.388	1.654	0.405
Gender (Male)	−4.328	1.210	<0.001
Age	0.045	0.102	0.665
ASIP gene R96C (R/C & C/C) and Age	0.023	0.198	0.909
ASIP gene R96C (R/C & C/C) and Gender (Male)	−1.261	1.487	0.400
Gender (Male) and Age	0.315	0.151	0.041

Table 6. Associations between lower back skin extension index (SEI) and *ASIP* SNP (R96C), gender, age, and two-way interactions, after stepwise linear regression analysis with backward elimination

	Lower back SEI		
	Estimate	Std. Error	*P*-value
Intercept	7.593	0.739	<0.001
ASIP gene R96C (R/C & C/C)	1.144	0.682	0.098
Gender (Male)	−4.420	0.095	<0.001
Age	0.051	1.191	0.595
Gender (Male) and Age	0.298	0.148	0.048

DISCUSSION

In order to investigate possible factors related to canine skin extensibility, we examined the association between SEI and some phenotype and genotype characteristics. First, univariable analysis indicated significant associations between lower back skin extension and dog gender, age and an *ASIP* polymorphism. Then, subsequent linear model analysis indicated that gender and *ASIP* SNP (R96C) affected lower back skin extension and that there was an association between gender and age. We think that this association might be due to the difference in the average age of the female and male individuals in the sample data; the mean female age was 6.95 (median=6), whereas the mean male age was 7.29 (median=7).

The deformability of skin depends on the relative amounts and viscoelastic properties of its individual components (including collagen, elastin and ground substance) [15]. Since a high SEI indicates a lack of collagen and reduced skin elasticity in dogs [27], it is possible that the higher skin extension in the female dogs was because they had lower collagen content than the males. Markova *et al.* (2004) reported that skin collagen content was significantly greater in maturing male mice than female mice [18]. Also, collagen was shown to decrease in orchiectomy rats, whereas it increased after testosterone was administered to female or castrated male rats [1]. Therefore, in our study the reason for the higher skin extension in the female dogs was probably because the female dogs were not able to suppress age-related collagen degradation by the anabolic action of male hormones such as testosterone.

However, although the skin extension index in neutered males with low androgen concentrations should therefore have been similar to the female dogs, we did not find any association between the neutering status and skin extension index. Two possible reasons for this lack of association are: 1) the gender factor might have masked the effect of neutering status on the skin extension index in our statistical models due to there being more neutered females than neutered males and 2) the average age at which the males and females were neutered might have been different or that neutering may have occurred long after puberty, both of which could have increased the androgen effect on skin extension. However, we could not confirm any association between low neutering age and high SEI because we did not have any data on neutering age of the dogs in this study.

The univariable analysis in this study, suggested a tendency of the association between the *ASIP* SNP (R96C) polymorphisms, which are thought to cause an Arginine to Cysteine substitution [12], and lower back skin extension (P=0.078). Furthermore, the linear model analysis also showed that *ASIP* SNP (R96C) tended to be associated with lower back skin extension (P=0.098). Also, there were no two-way interactions between *ASIP* SNP (R96C) and age and between *ASIP* SNP (R96C) and gender for lower back extension. In pigment cells, the *ASIP* protein binds to *MC1R* and has a competitive antagonist αMSH, but it also has various other actions in other cells.

Kim *et al.* (1994) reported that *ASIP* regulates several cellular functions, including Ca^{2+} signaling [13]. Also, it is possible that *ASIP* acts on pathways that target specific subtypes of Ca^{2+} channels [17]. Changes in Ca^{2+} are known to affect many processes, including hormone secretion and neurotransmitter release, enzyme and ion channel activity, and gene expression [5, 17]. Also, the distribution of Ca^{2+} in the epidermis plays a vital role in maintaining the skin barrier function including processes such as keratinocyte differentiation, skin barrier formation, and permeability barrier homeostasis [8, 14]. Various types of skin diseases characterized by barrier abnormalities or epidermal hyperplasia are thought to be associated with abnormal ionic dynamics [6]. Therefore, in the current study, it is possible that he *ASIP* SNP (R96C) could have been related to disruption of the Ca^{2+} balance that could have affected dermatitis and skin extensibility. However, due to the low number of samples in the study, it was not possible to show clearly whether there was a true association between *ASIP* SNP (R96C) and the lower back SEI (P=0.098). Therefore, in future studies the number of samples will need to be increased to confirm the association.

This is the first study to use SEI to indicate that gender could be related to canine skin extensibility. However, since there was no association between SEI and coat color, it suggests that coat color is not related to SEI or skin extensibility despite this being hypothesized by some veterinarians before the study began. Furthermore, our study suggests that apart from *ASIP* SNP (R96C), none of the other selected coat color candidate genes (*MC1R, TYRP1, MLPH, CBD103* and *MFSD12*) affect SEI or skin extensibility. However, since there were no samples in our study with abnormally high lower back skin extension, it was not possible to identify all the possible factors related to skin extensibility. Therefore, further studies are needed with more dogs to clarify all the factors that influence skin extensibility in dogs. When these factors are clarified, it should greatly contribute to the clinical diagnostics of skin extensibility in dogs. In future studies we will collect case samples with SEI values of more than 14.5%, and perform comparative analysis of cases and controls in order to better identify the factors involved in canine skin extensibility.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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