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Genomic association and further characterisation of faecal immunoglobulin A deficiency in German Shepherd dogs

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Abstract

Background: Immunoglobulin A (IgA) deficiency, chronic enteropathies and exocrine pancreatic insufficiency (EPI) have a high prevalence in German Shepherd dogs (GSD). This prospective study determined the prevalence of faecal IgA deficiency (IgAD) in GSD and investigated several candidate genes and the canine genome for a region or locus co-segregating with IgAD in GSD. Faecal IgA concentrations were quantified and genomic DNA was extracted from 8 GSD with an undetectable faecal IgA (classified as IgAD) and 80 non-IgAD GSD. The canine minimal screening set II microsatellite markers were genotyped, with evidence of an association at $p < 1.0 \times 10^{-3}$. Faecal IgA concentrations were also tested for an association with patient clinical and biochemical variables.

Results: Allele frequencies observed using the candidate gene approach were not associated with faecal IgAD in GSD. In the genome-wide association study (GWAS), the microsatellite marker FH2361 on canine chromosome 33 approached statistical significance for a link with IgAD in GSD ($p = 1.2 \times 10^{-3}$). A subsequent GWAS in 11 GSD with EPI and 80 control GSD revealed a significant association between EPI and FH2361 ($p = 8.2 \times 10^{-4}$).

Conclusions: The lack of an association with the phenotype of faecal IgAD in GSD using the candidate gene approach and GWAS might suggests that faecal IgAD in GSD is a relative or transient state of deficiency. However, the prevalence of faecal IgAD in GSD appears to be low (<3%). The relationship between faecal IgAD, EPI and loci close to FH2361 on canine chromosome 33 in GSD warrants further investigation.

KEYWORDS
calgranulin, canine trypsin-like immunoreactivity, faecal IgA, German Shepherd dog, intestine

1 | BACKGROUND

Immunoglobulin A (IgA) deficiency (IgAD) has been reported in German Shepherd dogs (GSD), and an increased risk for the development of chronic gastrointestinal disease has been associated with IgAD in this breed (Batt et al., 1991; German et al., 2000; Maeda et al., 2014). Mucosal secretion of IgA is a complex process (Figure 1) that requires the expression of genes encoding proteins involved in the synthesis, secretion and/or interaction of IgA molecules (Fagarasan & Honjo, 2003). In humans, 35 genes whose products are associated with the production and/or secretion of IgA have been associated with IgAD (Abolhassani et al., 2016). An impaired function of IgA-secreting plasma cells has been reported in GSD with IgAD (German et al., 2000, Littler et al., 2006, Asano et al., 2004). Studies on IgAD in canine idiopathic inflammatory bowel disease (IBD) have been primarily focused on the investigation of IgA production by plasma cells following class-switch recombination, proliferation, migration and differentiation of IgA^+ B cells (Lee et al., 2015; Nakazawa et al., 2019; Olsson et al., 2015) or epigenetic modification of mucosal IgA expression (Maeda et al., 2014), but have not specifically evaluated secretory IgA to determine the disease phenotype.

The *CARD15/NOD2* gene on canine chromosome 2 has been considered a potential candidate gene for faecal IgAD in GSD due to its central role in mediating innate immune responses (Roberts et al., 2006) and its association with chronic enteropathy in GSD (Kathrani et al., 2014). Investigating a microsatellite marker at a distance of about 2 megabases to the *CARD15/NOD2* gene (FH2608) revealed no association with IgAD in GSD (Tress et al., 2006a). However, a microsatellite marker located closer to this gene might show such an association. Several additional candidate genes were also considered, the products of which are involved in the synthesis and secretion of IgA. The *Q8MJZ1_CANFA* gene, located on chromosome 7 in dogs, presents a candidate gene for IgAD in GSD. This gene encodes the polymeric immunoglobulin receptor (PIgR) fragment containing the secretory component bound to secretory IgA (sIgA) (Johansen et al., 1999; Kaetzel et al., 1991). Another candidate gene localised on chromosome 13 in dogs is the immunoglobulin J-chain (*IGJ*) gene encoding for the immunoglobulin J-chain part of the IgA complex (Johansen et al., 2001; Sorensen et al., 2000). Both PIgR and IGJ are essential for the proper function of sIgA (Figure 1) (Mostov, 1994; Snoeck et al., 2006).

Four allelic variants (A–D) of the canine immunoglobulin alpha heavy chain (*IGHA*) gene were identified based on sequence variations in the hinge region, revealing a combination of single nucleotide polymorphisms (Peters et al., 2004). Currently, only one of those four allelic variants (variant C) of the gene encoding the *IGHA* in dogs has been identified in GSD (Peters et al., 2005). Hence, further research is warranted to investigate whether specific allelic variants of the canine *IGHA* gene are linked to IgAD in GSD.

The canine minimal screening set-2 (cMSS-2) encompasses a group of microsatellite markers that have been used in several studies to identify candidate gene regions of interest in canine hereditary diseases (Clark et al., 2006; Lippmann et al., 2007; Lowe et al., 2003). Genetic bottlenecks due to relative inbreeding led to long haplotype blocks in dogs and allow for association studies to evaluate for linkage by utilising small numbers of unrelated affected and unaffected individuals (Clark et al., 2005; Nolte & Te Meerman, 2002; Nordborg & Tavare, 2002). This technique is effective for studying genetic diseases in purebred dog populations (Awano et al., 2009; Clark et al., 2005; Hyun et al., 2003; Lippmann et al., 2007; Sutter et al., 2004).

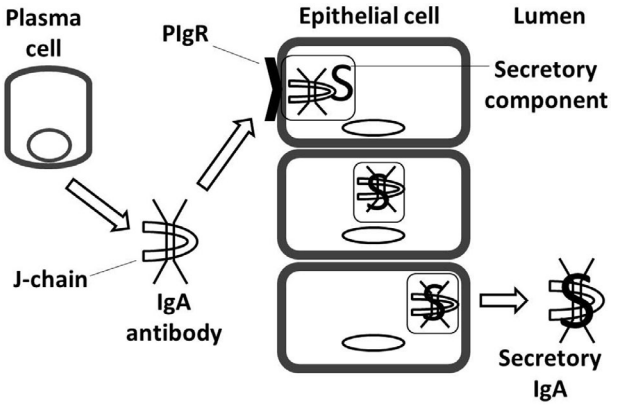

FIGURE 1 Synthesis and secretion of immunoglobulin A (IgA). This figure depicts the synthesis of secretory immunoglobulin A (sIgA) by intestinal epithelial cells following immunoglobulin A (IgA) production and secretion from differentiated plasma cells. The polymeric immunoglobulin receptor (pIgR) is expressed by mucosal epithelial cells and binds IgA dimers (via the J chain) at the basal part of these cells. After being transported through the epithelial cell, a portion of pIgR is cleaved from the IgA dimer rendering the secretory component with the resulting sIgA complex. Compared to humans and rodent species, canine IgA represents a dimer on mucosal surfaces and in plasma (Ellis, 2019)

Exocrine pancreatic insufficiency (EPI) is highly prevalent in GSD (Batchelor et al., 2007) and is diagnosed by an abnormally low serum canine trypsin-like immunoreactivity (cTLI) concentration (Wiberg et al., 1999). Dogs with EPI have an impaired secretion of endogenous antimicrobial substances produced in the pancreas, with the risk of developing small intestinal dysbiosis (SID; Simpson et al., 1989; Williams et al., 1987) reflected by alterations in serum folate and cobalamin concentrations (Suchodolski & Steiner, 2003). SID affects mucosal immunity (Kett et al., 1995; Riordan et al., 1997), including IgA responses (Alexander et al., 2014; Olsson et al., 2014). Calprotectin is a dimeric protein complex released from activated myelomonocytic cells and increases in serum and faecal samples from dogs with chronic intestinal inflammation (Grellet et al., 2013; Heilmann et al., 2012, 2018; Otoni et al., 2018). However, it has not been evaluated in GSD with IgAD or EPI.

The White Shepherd dog (WSD) is a breed that emerged from white-coated lines of GSD in the United States. Being a direct descendent of the GSD, the WSD is recognised as a separate breed only by the United Kennel Club (United Kennel Club Website, 2015). Faecal IgA, serum cobalamin, folate and cTLI concentrations have not been compared between GSD and WSD or male and female GSD.

We hypothesised that (1) microsatellite markers of the cMSS-2 and/or allelic variants of the *IGHA* gene are linked to faecal IgAD in GSD and (2) that EPI and IgAD are associated in GSD, with no difference in biochemical markers of both between GSD and WSD. Thus, the

TABLE 1 Total number, gender distribution and age of all dogs included in the study ($n = 132$)

	GSD			WSD			Total
	n	Female/age[a]	Male/age[a]	*N*	Female/age[a]	Male/age[a]	*n*
Genotyping							
Candidate genes							
IgAD	4	3/7.0	1/5.0	4	2/6.5	2/6.8	8
IgA within RI	50	32/5.0	18/3.0	30	18/3.0	12/2.0	80
GWAS							
IgAD	3	2/6.0	1/5.0	3	1/5.0	2/6.8	6
IgA within RI	15	8/5.5	7/3.0	15	9/7.0	6/4.5	30
EPI							
cTLI ≤2.5 μg/L	10	4/6.0	5/2.0	1	2/3.5	0/0.0	11
cTLI within RI	57	36/4.5	21/3.0	23	15/3.0	8/4.0	80
IGHA gene							
IgAD	2	1/4.0	1/5.0	3	1/5.0	2/6.8	5
IgA within RI	3	1/8.0	2/3.8	2	2/3.5	0/0.0	5
Faecal variables							
IgA	68	42/5.0	26/3.0	34	20/4.0	14/2.0	102
Calprotectin	34	20/6.0	14/2.3	13	8/2.0	5/6.0	47
Serum variables							
Normocobalaminaemia	68	42/5.0	26/3.0	34	20/4.0	14/2.0	102
Normofolatemia	68	42/5.0	26/3.0	34	20/4.0	14/2.0	102
Normal serum cTLI	68	42/5.0	26/3.0	34	20/4.0	14/2.0	102
Hypocobalaminaemia	12	8/6.0	4/3.0	9	6/3.3	3/5.0	21
Hypofolatemia	6	3/4.0	3/3.0	10	3/2.0	7/2.0	16
Equivocal cTLI (2.5–5.7 μg/L)	13	7/6.0	6/3.0	3	3/7.0	0/0.0	16
EPI (cTLI ≤ 2.5 μg/L)	10	4/6.0	6/2.0	2	2/3.5	0/0.0	12
Calprotectin	35	23/5.0	12/4.5	36	20/4.3	16/4.0	71

Abbreviations: cTLI, canine trypsin-like immunoreactivity; GSD, German Shepherd dogs; GWAS, genome-wide association study; IgA, immunoglobulin A; IgAD, IgA deficiency; *IGHA*, immunoglobulin alpha heavy chain; RI, reference interval; WSD, White Shepherd dog.
[a]Median age (in years).

objectives of this prospective case–control study were (1) to investigate the candidate genes *CARD15/NOD2*, *Q8MJZ1_CANFA*, *IGJ* and *IGHA*, and to perform a genome-wide association study (GWAS) using the cMSS-2 to identify a genomic region or locus that co-segregates with the phenotype of faecal IgAD in GSD; (2) to evaluate the relationship between faecal IgA concentrations and serum cTLI, folate, cobalamin and serum and faecal calprotectin concentrations in GSD; and (3) to evaluate the effect of breed line (GSD vs. WSD) and sex on faecal IgA and serum cTLI, folate and cobalamin concentrations.

2 | MATERIALS AND METHODS

2.1 | Sampling population and sample collection

Over 3 years (2005–2008), faecal, whole blood (or buccal mucosal swab) and serum samples were prospectively collected from pure-bred GSD and WSD from various parts of North America (Table 1). Owners of enrolled dogs were asked to complete a standard study questionnaire to obtain detailed information about the dogs' signalment and health status (including any current medications and vaccination status), with an emphasis on clinical signs suggestive of gastrointestinal disease. Four faecal specimens (approx. 1 g each) were collected from each day using a previously established sampling strategy: faecal samples were obtained from 2 consecutive days (days 1 and 2), and this was repeated 4 weeks later (days 28 and 29) (Tress et al., 2006b). Food was withheld from the dogs for ≥12 h before withdrawing a small amount of blood and collecting serum on day 28 or 29 of the faecal sampling protocol. A whole blood sample for subsequent genomic DNA analysis was obtained from 86 of the dogs and a buccal mucosal swab from 2 of the dogs. The protocol for collecting samples from GSD/WSD was reviewed and approved by the Clinical Research Review Committee at Texas A&M University (CRRC# 2005-35). Written consent was obtained from the owner prior to the enrolment of a dog in the study.

2.2 | Faecal and serum sample analyses

Faecal IgA was extracted and measured using a validated in-house sandwich enzyme-linked immunosorbent assay (ELISA) (Tress et al., 2006b). The reference interval (RI) for faecal IgA concentrations was a 4-day sample mean concentration of 0.22–3.24 mg/g faecal material, and IgAD was conservatively defined as an undetectable faecal IgA concentration (i.e., below the lower detection limit of 0.06 mg/g) for all four faecal samples (Tress et al., 2006b).

Serum cobalamin and folate concentrations were determined using automated chemiluminescence assays (Immulite®2000, Cobalamin/Folate, Siemens Healthcare Diagnostics, Inc., Deerfield, IL, USA). Serum cTLI concentration was measured using a competitive double-antibody RIA (^{125}I-cTLI RIA, Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The RI for serum cobalamin, folate and cTLI concentrations in dogs were 251–908 ng/L, 7.7–24.4 μg/L and 5.7–45.2 μg/L, respectively (Gastrointestinal Laboratory, Texas A&M University; http://vetmed.tamu.edu/gilab/service/assays; accessed October 2020). Serum and faecal canine calprotectin concentrations were measured in a subset of GSD and WSD using a validated in-house RIA (RI: 76–564 μg/L and <2.9–48.9 μg/g, respectively; Heilmann et al., 2008).

2.3 | Genomic DNA extraction and genotyping

Genomic DNA was isolated from 88 dogs using whole blood specimens (n = 86) or buccal mucosal swabs (n = 2) and a commercial kit (Puregene® DNA Isolation Kit, Gentra Systems, Minneapolis, MN, USA). The purity and quantity of the extracted DNA were evaluated spectrophotometrically prior to further analyses. Pedigrees of all dogs were evaluated for a minimum of two generations whenever possible to ensure that dogs were not closely related. A total of 88 unrelated pure-bred dogs (58 GSD and 30 WSD) were included in the genetic analysis using microsatellite markers (Table 1).

First, a candidate gene approach was chosen. The microsatellite markers FH2226 and FH3800 (both contained in the cMSS-2) were amplified by PCR, separated by capillary gel electrophoresis and analysed as previously described (Clark et al., 2004). Three additional microsatellite markers not included in the cMSS-2 and located in the regions flanking FH2608 on canine chromosome 2 (FH3280 and REN60B17) and FH3800 on canine chromosome 13 (C87704) were evaluated. All three microsatellite markers were amplified using fluorescent-labelled forward primers (6-FAM; Gene Technologies Lab, College Station, TX, USA) and unlabelled reverse primers, and were genotyped in all 88 dogs. Primer sequences were obtained from the canine genome assembly (CanFam2.0: http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9615&query; accessed August 2015).

Second, a subset of 36 unrelated pure-bred dogs (18 GSD and 18 WSD) (Table 1), from which a sufficient amount of genomic DNA was available, were included in a GWAS using the cMSS-2 (the method with the highest genomic coverage available at the time of the study) as previously described (Clark et al., 2004). Also, 11 GSD with serum cTLI concentrations diagnostic for EPI (cut-off value: ≤2.5 μg/L) and 80 unaffected (serum cTLI concentration within RI) GSD were genotyped using the complete cMSS-2 set. The most significant cMSS-2 microsatellite markers identified in the GWAS were then evaluated in the remaining dogs (52 for IgAD and 55 for EPI) that were not included in the GWAS due to an insufficient amount of genomic DNA available.

Third, to investigate whether certain allelic variants of the canine *IGHA* gene are linked to IgAD in GSD, genomic DNA from 10 GSD (five IgAD GSD and five control GSD) was analysed by fluorescence resonance energy transfer (FRET) melting temperature (Peters et al., 2004). The FRET dual-probe system was used to collect data on the accumulation of target DNA during real-time PCR, and nucleotide polymorphisms were identified by analysing the melting curve (Peters et al., 2004).

2.4 | Comparison of two faecal IgA ELISA and confirmation of faecal IgAD

Faecal IgA concentrations from nine GSD (three dogs classified as IgAD, one dog with faecal IgA in the questionable range and five dogs with a faecal IgA concentration with the RI) were re-analysed on a different IgA ELISA that uses a polyclonal goat anti-dog IgA as primary (capture) antibody and a monoclonal mouse anti-canine IgA as secondary (detection) antibody (Littler et al., 2006), and the results obtained from both ELISA systems were compared to verify the results. Also, follow-up faecal samples (collected as described) from all eight GSD with undetectable faecal IgA were attempted 2 years after study enrolment to verify the IgAD phenotype.

2.5 | Statistical analysis

All statistical analyses were performed using a commercially available statistical software package (GraphPad Prism®v5.0, GraphPad Software, San Diego, CA, USA). For each cMSS-2 microsatellite marker, the most frequent allele in the group of GSD with IgAD and the group of dogs with EPI was identified. This was compared to the respective allele frequency in dogs with a faecal IgA concentration within the RI and with a normal serum cTLI concentration, respectively. Fisher's exact test was used to evaluate the association between the two allele frequencies. Evidence of association in the GWAS was defined as $p < 1.0 \times 10^{-3}$ using Benjamini and Hochberg's false discovery rate for multiple testing (BenjaminiHochberg.xlsx; accessed May 2015). The relationship between *IGHA* gene allelic variants and IgAD in GSD was evaluated by Fisher's exact test.

A Mann–Whitney *U*-test was used to compare faecal IgA, serum cobalamin, folate, cTLI and serum and faecal calprotectin concentrations between GSD and WSD. Serum concentrations of cobalamin, folate and cTLI were also tested for a correlation with the mean faecal IgA concentration determined in samples collected on days 28 and 29 (n = 102) by calculating a Spearman rank sum correlation coefficient

TABLE 2 Results for the candidate gene approach

Candidate gene	Canine Chr	Microsatellite marker	Proximity to candidate gene (Mb)	Part of cMSS-2 set	Most frequent allele in IgAD GSD	Allele frequency in IgAD GSD	Allele frequency in control GSD	*p*-Value for association
CARD15/NOD2	2	FH3280	0.35	No	455	7/16 (44%)	42/160 (26%)	0.0759
		REN60B17	0.16	No	121	15/16 (94%)	107/160 (66%)	0.0166
Q8MJZ1_CANFA	7	FH2226	0.01	Yes	250	6/16 (38%)	12/160 (8%)	0.0019
IGJ gene	13	FH3800	3.40	Yes	405	7/16 (44%)	56/160 (35%)	0.1646
		C87704	0.00	No	113	8/16 (50%)	68/160 (43%)	0.6041

Note: Allele frequencies of microsatellite markers (FH2226, FH3800, FH3280, REN60B17 and C87704) for the candidate gene approach obtained from 8 GSD with IgAD and 80 GSD with a faecal IgA concentration within the reference interval. Columns indicate candidate genes; location on the canine genome (chromosome [Chr]); microsatellite marker evaluated; distance between microsatellite and candidate gene; microsatellite marker being part of the cMSS-2 (yes or no); most frequent allele (at least five different alleles per microsatellite marker); allele frequency observed in IgAD GSDs and control GSDs; and corresponding *p*-values for association (an adjusted *p*-value of $<1.0 \times 10^{-3}$ was considered statistically significant).
Abbreviations: cMSS, canine minimal screening set; GSD, German Shepherd dogs; IgA, immunoglobulin A; IgAD, IgA deficiency.

(ρ) with the 95% confidence interval (CI) for significant correlations. Fisher's exact test served to test the association between sex and IgAD and to compare the proportion of GSD and WSD with a serum cTLI concentration within the RI to those with a serum cTLI concentration below the RI (<5.7 μg/L) or below the cut-off for diagnosing EPI (≤2.5 μg/L). A Kruskal–Wallis test with Dunn's post-test was used to compare faecal IgA levels among GSD with a serum cTLI concentration diagnostic for EPI, serum cTLI concentration below the RI but above the cut-off for diagnosing EPI, and GSD with a serum cTLI concentration within the RI. A quadratic weighted kappa statistic served to evaluate the diagnostic agreement between the two faecal IgA ELISA tests (Cohen, 1968; Sim & Wright, 2005). The decision limits used were the respective RI for each ELISA (Tress et al. 2006b: 0.22–3.24 mg/g, Littler et al. 2006: >0.68 mg/g) and the lower detection limit of the assays (0.06 mg/g [Tress et al. 2006b] and 0.68 mg/g [Littler et al. 2006], respectively). The interpretation of the weighted kappa coefficient was based on a previous guideline [50]. Statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Sampling population

Faecal IgA concentrations were undetectable (i.e., a 4-day mean IgA concentration of <0.06 mg/g) in 8 of the 88 GSD (9%), and these dogs were classified as IgAD (Table 1). GSD with IgAD enrolled in this study frequently presented with clinical signs of otitis (38%) or gastrointestinal disease (i.e., diarrhoea, vomiting and/or weight loss; 25%). The 4-day mean faecal IgA concentrations in the remaining 80 GSD (91%) were within the RI and ranged from 0.23–2.98 mg/g (median: 0.76 mg/g). These 80 control GSD had no clinical signs reported to the investigators (NG, RMH).

GSD with IgAD were significantly older (median: 7.0 years, range: 4–10 years) than GSD with faecal IgA concentrations within the RI (median: 3.0 years, range: 1–12 years; $p = 0.0227$). Five of the eight GSD with IgAD (62%) were males and three of the dogs (38%) were females. Thirty (37%) of the GSD with normal faecal IgA concentrations were males and 50 (63%) were females; sex and IgAD were not significantly associated ($p = 0.3025$).

3.2 | Genotyping

Allele frequencies of the canine microsatellite markers FH2226, FH3800, FH3280, REN60B17 and C87704 for the candidate approach were not significantly different between GSD with IgAD ($n = 8$) and those dogs with faecal IgA concentrations within the RI ($n = 80$; for all $p > 1.0 \times 10^{-3}$) (Table 2).

A total of 294 microsatellite markers (90%) of the cMSS-2 were genotyped across all 38 autosomes and the 2 sex chromosomes in all 36 unrelated dogs included in the GWAS (6 dogs classified as IgAD and 30 control dogs). Fourteen markers (4%) of the cMSS-2 could not be amplified, and 19 of these markers (6%) could not be consistently genotyped. Amplicons of those 19 microsatellite markers showed a broad peak spectrum with non-distinct allele values. The GWAS using the cMSS-2 revealed no significant association for any of the 294 microsatellite markers with IgAD. However, the *p*-value obtained for one microsatellite marker (FH2361 located on canine chromosome 33 at position 19,158,127–19,158,477 [bp]) approached statistical significance ($p = 1.2 \times 10^{-3}$) (Figure 2). Allele 351 of FH2361 occurred with a frequency of 11 of 16 possible alleles (69%) in 8 dogs with IgAD and 44 of 160 alleles (28%) in 80 control dogs (Table 3A). For the remaining 293 microsatellite markers, no *p*-value was below 1.2×10^{-3}.

The second GWAS using the cMSS-2 revealed that in 11 GSD with a cTLI concentration diagnostic for EPI (Table 1), allele 340 of marker FH2361 (the marker with the lowest *p*-value in the IgAD GWAS) occurred with a frequency of 7 of 22 possible alleles (32%) compared to 9 of 160 alleles (6%) in 80 GSDs with a cTLI concentration within the RI (Table 3B). The resulting *p*-value for FH2361 (8.2×10^{-4}) indicated a significant association with EPI.

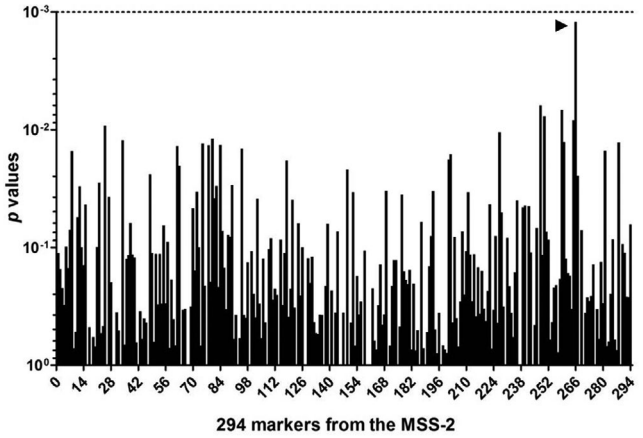

FIGURE 2 Results of the genome-wide association study (GWAS). This figure represents the individual *p*-values for all 294 microsatellite markers computed for the genome-wide scan. The arrow indicates the lowest (but non-significant) *p*-value of microsatellite marker FH2361; the dashed line defines the significance level for this study using the Benjamini and Hochberg's false discovery rate for multiple testing

TABLE 3 Allele frequencies of microsatellite marker FH2361

(A)	IgAD GSD	Control GSD	Σ
Allele 351	11	44	55
Other alleles	5	116	121
Σ	16	160	176
(B)	**EPI GSD**	**Control GSD**	**Σ**
Allele 340	7	9	16
Other alleles	15	151	166
Σ	22	160	182

Note: Contingency table summarising the observed allele frequencies of microsatellite marker FH2361 in GSD: (A) 8 GSD with IgA deficiency (IgAD GSD) and 80 GSD with faecal IgA concentrations within the reference interval (Control GSD), $p = 1.2 \times 10^{-3}$; (B) 11 GSDs with exocrine pancreatic insufficiency (EPI GSDs) and 80 GSDs with serum cTLI concentrations within the reference interval (Control GSDs), $p = 8.2 \times 10^{-4}$. Σ = row or column sum.

Abbreviations: cTLI, canine trypsin-like immunoreactivity; EPI, exocrine pancreatic insufficiency; GSD, German Shepherd dogs; IgAD, IgA deficiency.

FRET analysis of *IGHA* revealed a single melting temperature for all 10 GSD evaluated, consistent with homozygosity for variant C. An association between IgAD and canine *IGHA* gene variants in GSD was not identified in this study.

3.3 | Serum and faecal variables in GSD and WSD

Faecal IgA concentrations in hypocobalaminaemic GSD (serum cobalamin concentration <251 ng/L, $n = 21$) were significantly higher (median: 1.19 mg/g) than in normocobalaminaemic GSD (median: 0.62 mg/g, $n = 102$; $p = 0.0372$) (Table 1). Faecal IgA concentrations were not different between hypofolatemic GSD (serum folate concentrations <7.7 μg/L; median: 0.48 mg/g; $n = 16$) and normofolatemic GSD (median: 0.31 mg/g; $n = 102$; $p = 0.3722$).

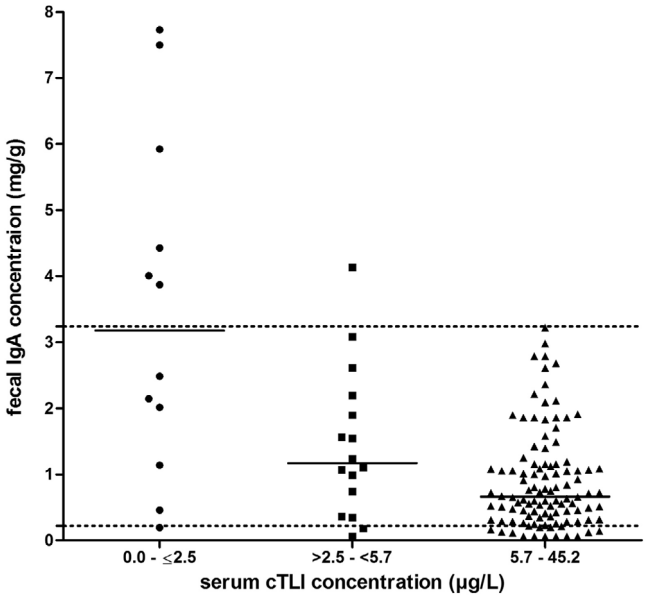

FIGURE 3 Association between faecal IgA and serum cTLI concentrations. Scatter plot of faecal IgA concentrations in 102 GSD with a serum cTLI concentration within the reference interval (median: 0.66 mg/g), 16 GSD with equivocal serum cTLI concentration (below the reference interval but above the cut-off for a diagnosis of EPI; median: 1.17 mg/g) and 12 GSD with cTLI concentrations diagnostic for EPI (≤2.5 μg/L; median: 3.18 mg/g). Faecal IgA concentrations differed significantly among the three groups of GSD ($p = 0.0002$), with GSD with a serum cTLI concentration diagnostic for EPI having significantly higher faecal IgA concentrations than GSD with a serum cTLI within the reference interval ($p = 0.0002$). The dashed horizontal lines indicate the reference interval for faecal IgA concentration (0.22–3.24 mg/g). cTLI, canine trypsin-like immunoreactivity; EPI, exocrine pancreatic insufficiency; GSD, German Shepherd dogs; IgA, immunoglobulin A

Faecal IgA concentrations were compared between GSD with a serum cTLI concentration within the RI ($n = 102$), GSD with equivocal serum cTLI concentration (below the RI [<5.7 μg/L] but above the cut-off value for the diagnosis of EPI [2.5 μg/L]; $n = 16$) and GSD with a cTLI concentration diagnostic for EPI (≤2.5 μg/L; $n = 12$) (Table 1). Faecal IgA concentrations differed significantly among these 3 groups of GSD ($p = 0.0002$). The post-test showed significantly lower faecal IgA concentrations in the group of GSD with a serum cTLI concentration within the RI (median: 0.66 mg/g) than in GSD with cTLI consistent with a diagnosis of EPI (median: 3.18 mg/g, $p = 0.0002$) (Figure 3).

Evaluation of the relationship between the 2-day sample mean faecal IgA concentration (days 28 and 29; median: 0.66 mg/g; range: 0.06–5.58 mg/g) and age (median: 3.0 years; range: 1.0–13.0 years), serum cobalamin (median: 421 ng/L; range: 250–895 ng/L), folate (median: 11.8 μg/L; range: 3.9–23.3 μg/L) and cTLI concentration (median: 9.4 μg/L; range: 5.8–30.6 μg/L) in 102 GSD (40 males, 62 females; Table 1) showed that the 2-day mean faecal IgA concentrations were not correlated with age, serum cobalamin, folate, nor cTLI

concentrations (all $p > 0.05$). Serum calprotectin (median: 705, range: 29–4001; $n = 72$) and serum folate concentrations were weakly positively correlated ($\rho = 0.2795$, 95% CI [0.0423, 0.4868]; $p = 0.0183$), and a moderate inverse relationship existed between serum calprotectin and serum cTLI concentrations ($\rho = -0.4340$, 95% CI [−0.6104, −0.2166]; $p = 0.0002$). Nine of the 47 GSD in which faecal calprotectin concentration was measured (18%) had a faecal calprotectin concentration above the RI, whereas 44 of the 71 GSD in which the serum calprotectin concentration was determined (61%) were found to be hypercalprotectinaemic. Faecal calprotectin concentrations (median: 11.7, range: 2.9–567.2; $n = 49$) were not correlated with faecal IgA, and serum folate, cobalamin and cTLI concentrations (all $p < 0.05$).

3.4 | Comparison of serum and faecal variables between GSD and WSD

Faecal IgA concentrations were not significantly different between GSD ($n = 68$; median: 0.73 mg/g) and WSD ($n = 34$; median: 0.29 mg/g; $p = 0.1577$), and IgAD was not significantly more frequently detected in any of the two populations of shepherd dogs ($p = 0.4438$) (Table 1). Sex-associated differences in faecal IgA concentrations were not identified in GSD (male: median = 0.80 mg/g; female: median = 0.69 mg/g) or WSD (male: median = 1.05 mg/g; female: median = 0.52 mg/g; for all $p > 0.05$). Also, faecal IgA concentrations did not differ among dogs of different age groups (1–2 years [$n = 33$, median: 0.67 mg/g], 3–6 years [$n = 43$, median: 0.75 mg/g] and ≥7 years [$n = 26$, median: 0.41 mg/g]; $p = 0.1083$).

Serum cobalamin concentrations were significantly higher in GSD (median: 438 ng/L) than in WSD (median: 355 ng/L; $p = 0.0163$), but serum folate concentrations did not differ between GSD (median: 12.1 μg/L) and WSD (median: 10.8 μg/L; $p = 0.0708$). GSD had significantly lower serum cTLI concentrations (median: 8.7 μg/L) than WSD (median: 10.5 μg/L; $p = 0.0091$). However, the differences in serum cobalamin and cTLI concentrations were not considered clinically relevant as they were within the respective RI for all dogs, and EPI was not significantly more common in GSD (11%) than in WSD (5%; $p < 0.05$). Serum calprotectin concentrations did also not differ between GSD (median: 725 μg/L) and WSD (median: 586 μg/L; $p = 0.1658$), and this result was mirrored for the comparison of faecal calprotectin concentrations between GSD (median: 11 μg/g) and WSD (median: 16 μg/g; $p = 0.9909$).

3.5 | Follow-up faecal IgA testing and comparison of two faecal IgA ELISA

Not finding a genetic marker co-segregating with phenotypic IgAD required verifying the IgAD phenotype over time in the eight GSD with undetectable faecal IgA. Owners of these eight dogs were asked to resubmit four faecal samples again from their dog 2 years after the initial sample submission. Four of the eight GSD (50%) initially categorised as faecal IgAD were still alive, and the owners of three of those dogs (75%) agreed to re-testing. Faecal IgA was extracted and quantified in these three GSD: one GSD (33%) still had undetectable faecal IgA (i.e., <0.06 mg/g) in all four samples, whereas faecal IgA concentrations were within the lower 25th percentile of the RI in the two remaining dogs (67%) (Table 4).

TABLE 4 Summary of the faecal IgA quantification in IgAD GSD ($n = 8$) in the study

GSD	Initial testing (mg/g) (2005)	Re-testing (2007)
1	0.06	N/A[a]
2	0.06	N/A[a]
3	0.06	N/A[a]
4	0.06	N/A[a]
5	0.06	N/A[b]
6	0.06	0.06 mg/g
7	0.06	0.41 mg/g
8	0.06	0.92 mg/g

Abbreviations: GSD, German Shepherd dogs; N/A, not applicable.
[a]Not alive.
[b]Re-testing not consented to by owner.

Lack of a persistent IgAD phenotype over time in two of the three GSD also required verifying the results for the initial extracts of all four faecal samples from nine GSD on a different IgA ELISA (Littler et al., 2006): three dogs previously classified as IgAD (i.e., <0.06 mg IgA/g faecal material in all four samples), one dog with equivocal faecal IgA concentrations (i.e., a 4-day mean faecal IgA concentration between 0.06 and 0.22 mg/g) and five dogs with a 4-day mean faecal IgA concentration within the RI (0.22–3.24 mg/g). The minimum detectable faecal IgA concentration (i.e., a colour signal equal to that of the mean plus 2 standard deviations of 20 blank samples) determined for the in-house ELISA (Tress et al., 2006b) and the ELISA used by Littler et al. (2006) was 0.06 mg/g and 0.68 mg/g, respectively. Considering these minimum detectable faecal IgA concentrations, a comparison of the faecal IgA concentrations yielded identical results for 3 GSD with faecal IgAD and 5 GSD with faecal IgA concentrations within the RI (Table 5). However, the ELISA used by Littler et al. (2006) did not detect any IgA in all four faecal extracts from the GSD, with faecal IgA concentrations in the questionable range using the in-house ELISA (Tress et al. 2006b). A very strong agreement was detected between both assays (Cohen's $\kappa = 0.795$) (Table 6).

4 | DISCUSSION

IgAD has been reported to occur in several different breeds, such as the Beagle, Chinese Shar-Pei, Irish Wolfhound, Cocker Spaniel, Chow Chow and GSD (Batt et al., 1991; Felsburg et al., 1985; Moroff et al., 1986; Olsson et al., 2014). In GSD, IgA concentrations have been investigated in lacrimal fluid (Day, 1996) and faeces (Littler et al., 2006), but those particular studies have not definitively documented IgAD in this breed. Other studies that evaluated the concentration of

TABLE 5 Faecal IgA concentrations measured using two different ELISA systems

Dogs	Faecal IgA (mg/g) Concentration (A)	Faecal IgA (mg/g) Concentration (B)
1	0.06[a]	0.68[a]
2	0.06[a]	0.68[a]
3	0.06[a]	0.68[a]
4	0.12[b]	0.68[a]
5	0.37[c]	1.26
6	0.51[c]	0.83
7	0.40[c]	0.81
8	0.34[c]	0.85
9	0.30[c]	0.92

Note: Assay (A): Tress et al. (2006b); assay (B): Littler et al. (2006).
Abbreviation: IgA, immunoglobulin A.
[a]Corresponding minimally detectable faecal IgA concentration.
[b]Faecal IgA concentration within the questionable range.
[c]Faecal IgA concentrations within the reference interval.

immunoglobulin subtypes in serum, saliva and tears also did not find a difference between healthy and diseased GSD or healthy and diseased dogs of other breeds (German et al., 1998). However, lower serum IgA concentrations have been documented in GSD compared to dogs of other breeds (Batt et al., 1991; Olsson et al., 2014). The hypothesis of serum IgA concentrations in GSD reflecting intestinal IgA production and secretion was disproven by German et al. (1998) and Rinkinen et al. (2003) who found that serum IgA concentrations are not correlated with the intestinal mucosal secretion of IgA. Also, the numbers of IgA-producing cells in GSD and other breeds were similar (German et al., 1998), but decreased numbers of IgA^+ cells in the intestinal lamina propria were detected in dogs with IBD (Lee et al., 2015). This is not surprising because serum IgA is an inflammatory antibody, interacting with IgA receptors, while sIgA has anti-inflammatory features. Thus, systemic and mucosal IgA appear to be part of different arms of the inflammatory response (Monteiro & van de Winkel, 2003; Otten & van Egmond, 2004). In contrast to previous studies (Olsson et al., 2014, 2015), our study did not consider serum IgA concentrations but used sIgA concentrations in faecal samples to define the phenotype of IgAD.

Alleles of several microsatellite markers close to three candidate genes, including *CARD15/NOD2* on canine chromosome 2, were not linked to phenotypic faecal IgAD in GSD in this study. Neither the microsatellite marker FH2608, which was previously found not to be linked to IgAD in GSD (Tress et al., 2006a), nor the markers FH3280 and REN60B17 were associated with faecal IgAD in GSD in the present study. FH2226, the marker closest to the candidate gene *Q8MJZ1_CANFA* located on canine chromosome 7, and FH3800 and C87704, closest to the *IGJ* gene, were also not associated with faecal IgAD in GSD. The GWAS using the cMSS-2 did not detect significance for any of the 294 microsatellite markers for faecal IgAD in GSD that were successfully evaluated, although the *p*-value obtained for one marker (FH2361) located on canine chromosome 33 approached statistical significance. Candidate genes in close proximity (within 700 base pairs) to FH2361 are *LSAMP* (limbic system-associated membrane protein), *GAP43* (growth-associated protein-43) and *TIGIT* (T-cell immunoreceptor with Ig and ITIM domains). Thus, further investigation of these candidate genes, particularly *TIGIT*, is warranted. Our results disagree with another GWAS in GSD that identified several genomic loci with candidate genes on canine chromosome 5 (*KIRREL3*), chromosome 8 (several genes, e.g., *GSC*, *PPP4R4* and *SERPINA9*) and chromosome 23 (*GPR149*) (Olsson et al., 2015). Possible explanations for this discrepancy are the differences in GWAS methodology and/or study population, the latter of which was affected by a slight selection bias in the previous study (Olsson et al., 2015). Genetic fixation resulting from genetic bottlenecks such as that shown for *SLIT1* on chromosome 28 in GSD (Olsson et al., 2015) can affect the phenotypic variance and is an additional or alternative explanation for the lack of a significant association in our GWAS. Similar to others (Olsson et al., 2015), the GWAS also identified no association of faecal IgAD with genomic loci in the major histocompatibility complex (MHC) region, which is associated with IgAD in humans (Abolhassani et al., 2016; Ellis, 2019; Vo Ngoc et al., 2017).

Allelic variants of the canine *IGHA* gene were previously evaluated by FRET, which identified four allelic variants (A–D) of the *IGHA* gene based on a combination of single nucleotide polymorphisms causing sequence variation within the hinge region (Peters et al., 2004, 2005). A relationship between the susceptibility of GSD to IgAD and canine *IGHA* gene variants present within this breed was not found in this study. Analysis of *IGHA* revealed a single melting temperature for all GSD consistent with homozygosity for variant C, the genotype present in most GSD evaluated by FRET (Peters et al., 2005).

Lack of an association of any of the markers investigated using the candidate gene and GWAS approach with the phenotype of faecal IgAD in GSD suggests that faecal IgAD in GSD might be a more complex genetic, polygenic or multifactorial disorder, which is consistent with the results of another genomic study (Olsson et al., 2015). Another possibility is that faecal IgAD in GSD might represents a relative or transient condition associated with a regulatory defect rather than an absent IgA production and/or secretion, thus producing a variable or fluctuating phenotype. This possible explanation is consistent with previous studies on serum IgA in GSD (Olsson et al., 2014) and also our findings with repeat faecal IgA determinations in dogs classified as IgAD.

Association studies are crucially dependent on the correct assignment of well-defined phenotypes. In addition to patient characteristics and biochemical markers, information about the health status of each dog was extracted from a standard study questionnaire and confirmed that control dogs enrolled in the study were healthy. The faecal IgA assay developed by Tress et al. (2006b) yielded comparable results to the ELISA used by Littler et al. (2006) for categorising GSD as IgAD or healthy. Still, measuring faecal IgA concentrations might not be sufficient for a definitive diagnosis of IgAD in GSD.

Re-testing of faecal IgA concentrations in a very small group of GSD ($n = 3$) 2 years after initial enrolment in the study revealed a low-normal faecal IgA concentration in two of three GSD (67%) that had

TABLE 6 Agreement between the two different faecal ELISA systems by using the nine GSD (Table 5)

	Faecal IgA ELISA (Tress et al., 2006b)				
		Below assay detection limit[a]	Within questionable range[b]	Within reference interval[c]	%
Faecal IgA ELISA (Littler et al., 2006)	Below assay detection limit[d]	**3**	**1**	**0**	*44*
	Within reference interval	**0**	**0**	**5**	*56*
	%	*33*	*11*	*56*	*100*

Note: Cohen's κ (quadratic weighted) = 0.795 (95% CI [0.466, 1.000]); very good agreement ($p = 0.002$); observed agreement = 0.889 (95% CI [0.581, 0.831]); chance agreement = 0.457.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IgA, immunoglobulin A.

[a] <0.06 mg IgA/g faeces.

[b] 0.06–0.22 mg IgA/g faeces.

[c] 0.22–3.24 mg IgA/g faeces.

[d] <0.68 mg IgA/g faeces.

previously been classified as IgAD. In contrast, only one GSD (33%) still had an undetectable faecal IgA concentration, which was an unexpected finding. However, these results are in line with other studies (Batt et al., 1991; German et al., 2000; Littler et al., 2006) and might suggest that faecal IgAD in GSD may not represent a permanent, but rather a relative or transient condition in GSD. However, there is currently no consensus about the cut-off concentration used to distinguish low faecal IgA concentrations and faecal IgAD in GSD, and the RI used in this study was determined from dogs of various breeds (Tress et al., 2006b). It should also be noted that the initial faecal IgA results were reported to the owners, and this may have influenced the owners' decision to use supplements, medications, dietary and/or lifestyle interventions to improve their dog's immune system. This practice may have affected faecal IgA concentrations at the time of re-testing because IgA responses can be affected by several factors (e.g., dietary changes, stress, antibiotic administration, intestinal dysbiosis and pathogen shedding; Abolhassani et al., 2016; Alexander et al., 2014; Grellet et al., 2016; Kikkawa et al., 2003; Maria et al., 2017; Svobodová et al., 2014; Vo Ngoc et al., 2017).

The lack of overt clinical signs in the majority of GSD with IgAD in this study (38% of GSD classified as IgAD had an ear infection and 25% had diarrhoea, vomiting and/or weight loss) is similar to humans and in contrast to other immunodeficiencies (Vo Ngoc et al., 2017), further supporting the hypothesis of a relative IgAD that could be secondary to another disorder. The fact that GSD with IgAD were significantly older than GSD with faecal IgA concentrations within the RI may also suggest that IgAD represents a hereditary disease process that manifests later in life. In contrast to puppies that produce less IgA due to immaturity of the mucosal immune system, age per se does not or only negligibly affect faecal IgA concentrations in adult dogs (Grellet et al., 2013, 2016; Olsson et al., 2014, 2015; Zaine et al., 2011).

During the development and validation phase of our in-house faecal IgA ELISA (Tress et al., 2006b) and the screening of GSD for the current IgAD study, we collected faeces from approximately 300 GSD. Of those 300 GSD, faecal IgA was initially undetectable in only 8 dogs (2.7%) and was consistently undetectable in only one dog (0.3%). This low prevalence of IgAD was unexpected and, as a limitation of this study, might not be sufficient for linkage analysis. However, five of the eight dogs (63%) were not available for re-testing to confirm the IgAD phenotype. Also, the two dogs that did not appear to have faecal IgAD when re-tested 2 years later still only had a low-normal faecal IgA concentration, supporting the theory of a "fluctuating phenotype" of IgAD in GSD (i.e., faecal IgA varying between undetectable and low-normal concentrations) as suggested based on systemic IgA levels in GSD (Olsson et al., 2014, 2015).

A high prevalence of EPI, mostly due to pancreatic acinar atrophy, is reported in GSD (Batchelor et al., 2007). This disease is characterised by a decreased secretion of pancreatic enzymes and other secretory components, including endogenous antibacterial compounds, and can cause secondary SID and affect IgA responses (Batchelor et al., 2007; German et al., 2000; Simpson et al., 1989, 1990; Williams et al., 1987). SID often occurs secondary in dogs (e.g., with EPI or IBD) but may be idiopathic in some breeds (Rutgers et al., 1995; Simpson et al., 1990). We found no difference in the prevalence of EPI between GSD and WSD in this study. However, dogs with a decreased serum cTLI (i.e., below the RI or cut-off value for diagnosing EPI) had significantly higher faecal IgA concentrations than dogs with a normal serum cTLI concentration (i.e., within the RI). This finding is consistent with an increased number of intestinal IgA-producing cells in non-GSD with SID (German et al., 2001) but contrasts with the results of studies showing decreased systemic IgA levels in GSD with pancreatic acinar atrophy (Olsson et al., 2014, 2015) and no difference in the number of small intestinal lamina propria IgA-producing cells in GSD with SID (Batt et al., 1991). In humans, increased numbers of lamina propria IgA-producing plasma cells are also reported with SID (Riordan et al., 1997) and increased serum sIgA levels with chronic pancreatitis (Frulloni et al., 2000). Finding an association of microsatellite marker FH2361 on canine chromosome 33 that was significant for EPI and approached statistical significance for faecal IgAD in GSD, further supports a link between compromised exocrine pancreatic function (EPI) and alterations in IgA responses (Olsson et al., 2014, 2015). Diseases associated with decreased IgA levels often produce overactive immune responses because IgA function involves no overt inflammation and decreases mucosal damage (Lee et al., 2015; Maeda et al., 2013). Hence, decreased faecal IgA concentrations in GSD with EPI may be a cause or consequence of intestinal immune dysfunction, disruption

of intestinal barrier function and/or changes in the composition of the intestinal microbiome. Further studies are warranted to characterise this association better.

GSD have a high prevalence of primary inflammatory (immune-mediated) diseases, including idiopathic IBD (Batt et al., 1991; Edwards et al., 1995; Rutgers et al., 1988, 1995), and IgA is important for maintaining the integrity of the intestinal mucosal barrier (Ellis, 2019). In humans, selective IgAD is the most common primary immunodeficiency and is also linked to chronic gastrointestinal disease (Cunningham-Rundles, 2001). Such an association with relative or absolute IgAD may be relevant in GSD, given the increased susceptibility of this breed to chronic gastrointestinal diseases (Allenspach, 2011) and decreased faecal IgA concentrations in dogs with IBD (Maeda et al., 2013; Nakazawa et al., 2019; Olsson et al., 2014). As a marker of inflammation, serum and faecal calprotectin concentrations are increased in dogs with IBD (Grellet et al., 2013; Heilmann et al., 2012, 2018; Otoni et al., 2018). Approximately 61% of the dogs in this study had an increased serum, and 18% an increased faecal calprotectin concentration, with no differences between GSD and WSD. GSD were more likely to be hypercalprotectinaemic than WSD, but this difference did not reach statistical significance ($p = 0.0544$; data not shown). These results suggest the existence of an inflammatory phenotype in some of these dogs, with a lower tendency for WSD than in GSD. This is consistent with a previous study identifying a connectivity between genes involved in the inflammatory response and linked to varying IgA levels in GSD (Olsson et al., 2015). The positive relationship between serum calprotectin and folate concentrations also suggests a link between changes in the small intestinal microbiome (primary or secondary SID) and an increased number or activity of inflammatory cells. However, future studies need to prove this hypothesis and investigate serum and faecal calprotectin concentrations in GSD with low IgA concentrations or IgAD.

We acknowledge that a limitation of our study is the relatively small number of dogs included. A larger number of dogs ($n > 300$) may have allowed to identify a putative association that was not observed here.

5 | CONCLUSIONS

In this study, neither alleles of microsatellite markers close to certain candidate genes nor a genomic region or locus within the entire canine genome were found to be associated with phenotypic faecal IgAD in GSD. However, one microsatellite marker located on chromosome 33 (FH2361, significantly associated with EPI) approached significance for an association with IgAD in GSD. While the physiology of IgA expression and secretion is well understood, it remains to be further studied whether faecal IgAD in GSD is an absolute or relative, permanent or transient, primary or secondary condition, and whether it is a hereditary disorder. The results of our study might suggest IgAD in GSD to be a relative or transient state of deficiency that could be a complex genetic or multifactorial condition or could be the immunological phenotype of an underlying disease (e.g., EPI or IBD). Also, the screening of approximately 300 GSD for our study suggests the prevalence of IgAD in GSD to be low (<3%). Further studies are warranted in GSD to explore the relation between faecal IgAD, EPI, idiopathic IBD and genomic loci in close proximity to FH2361 on canine chromosome 33.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

NG: research design, conduct of the study, data analysis, manuscript preparation; RMH: research design, conduct of the study, data analysis, manuscript preparation; UT: research design, sample preparation, manuscript revision; IRP: research design, conduct of the study, manuscript revision; JSS: research design, manuscript revision; JMS: research design, manuscript revision.

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