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# Malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in canine serum: establishing reference intervals and influencing factors

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## Abstract

**Background** Mounting evidence suggests that malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) are valuable biomarkers of lipid and nucleic acid oxidation in numerous canine diseases. However, their application in clinical settings is limited due to the absence of reference intervals (RI) and the analytical inconsistencies. Therefore, this study aimed to characterize serum MDA and 8-OHdG concentrations in dogs, to establish assay-specific RI, and to identify biological, haematological and biochemical factors influencing these markers.

**Methods** A total of 190 clinically healthy dogs were recruited, including pet dogs, working dogs and shelter dogs. Serum MDA concentration was measured by the Thiobarbituric Acid Reactive Substances (TBARS) assay, while 8-OHdG levels were determined by using a competitive ELISA. RI were established by non-parametric methods. Potential associations between oxidative stress (OS) biomarkers and multiple biological, haematological and biochemical factors were assessed using multivariate regression models.

**Results** RI for serum MDA (1.85–14.51 µM) and 8-OHdG (0.06–0.75 ng/mL) were established in the reference population (144 and 143 dogs, respectively). The multivariate regression model for MDA revealed a positive association with total cholesterol concentration, and a negative association with monocyte count. 8-OHdG level was positively associated with urea concentration. Notably, both models also revealed a significant association between MDA and 8-OHdG. Biological factors, including the age and size of the animals, did not exert a significant influence on the results.

**Conclusions** This is the first study to establish serum RI for MDA and 8-OHdG in a large and diverse canine population. Additionally, the multivariate regression models identified relevant haematological and biochemical, but not biological factors that should be considered when interpreting the results. These findings could significantly

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enhance the application of MDA and 8-OHdG as biomarkers in clinical settings, and promote further exploration of their value in canine diseases.

**Keywords** Dog, Guanine oxidation, Lipid peroxidation, Normal, Oxidant, Range

## Background

The term "oxidative stress" (OS) was first introduced by Helmut Sies in 1985 as an imbalance between oxidants and antioxidants, favouring the oxidants, that may induce damage in biological systems [1]. The concept has undergone redefinition over the years to account for its essential role in physiological signaling processes. Nevertheless, it has been proven to participate in the pathogenesis of multiple diseases, as a consequence of the molecular damage caused by the accumulation of excessive amounts of reactive species, especially reactive oxygen species (ROS), reactive nitrogen species (RNS), and their byproducts [2–4].

Given that redox biology involves numerous compounds and biochemical pathways, a major challenge in assessing OS in clinical practice lies in its measurement [4–6]. Diverse biomarkers have been identified, reflecting either direct oxidative damage to biomolecules or the antioxidant defences. Oxidative damage can be assessed by measuring products of ROS-mediated modifications. Malondialdehyde (MDA), a metabolite of lipid peroxidation, is one of the most widely employed biomarkers of OS. Numerous techniques have been developed for its quantification, including High Performance Liquid Chromatography (HPLC) and mass spectrometric assays, which display high sensitivity and specificity. However, the Thiobarbituric Acid Reactive Substances (TBARS) assay is one most commonly used assays due to its simplicity and availability in clinical settings [7–11]. Among DNA oxidation biomarkers, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one of the most extensively studied. 8-OHdG is a byproduct of ROS-mediated guanine oxidation, which can be quantified in tissues and body fluids by various highly specific but complex techniques (e.g. HPLC, gas chromatography with mass spectrometry) as well as by more readily available assays such as Enzyme-Linked Immunosorbent Assay (ELISA) [12–16].

These biomarkers have demonstrated clinical utility in dogs. Elevated MDA levels, as measured by the TBARS assay, have been associated with various canine diseases, including leishmaniosis [17–19], ehrlichiosis [20, 21], hypothyroidism [22, 23], inflammatory bowel disease [24], chronic kidney disease [25], obesity [26], hyperlipidaemia [27], and cancer [9, 28]. MDA has also been found to be useful for assessing other situations in dogs such as emotional stress, recovery after surgery and physical exercise, among others [29–36]. Furthermore, increased DNA oxidation, as indicated by elevated 8-OHdG levels, has been observed in dogs with babesiosis [37], atrial fibrillation [38], malignant mammary gland tumours [39] and following prolonged exercise [40].

However, despite the evidence of the clinical value of MDA and 8-OHdG in canine diseases, their use in veterinary medicine remains largely confined to research. Several factors contribute to this, including the lack of established reference intervals (RI) and the variability in analytical assays. Currently, no established RI have been reported for 8-OHdG in dogs, and only one study has suggested RI for plasma MDA in this species, using a relatively small sample size [41]. Additionally, multiple analytical assays can be used to measure MDA and 8-OHdG, each with inherent variations. This variability can significantly impact the reported values, making comparison between studies and individual cases difficult [6, 7, 11, 41], and limiting their application in clinical practice.

Hence, the main objective of this study was to characterize serum concentrations of MDA and 8-OHdG in healthy dogs. We aimed to establish assay-specific RI for MDA measured by TBARS, and 8-OHdG measured by competitive ELISA, in accordance to the American Society for Veterinary Clinical Pathology (ASVCP) guidelines [42]. Additionally, we assessed the influence of various sources of biological variation on the assays, and the relationship between these biomarkers and several haematological and biochemical variables. Providing data on these aspects could pave the way for the application of these biomarkers in clinical settings.

## Methods

### Reference population, inclusion and exclusion criteria

This study followed the ASVCP's Quality Assurance and Laboratory Standards Committee (QALS) guidelines for the determination of *de novo* RI in veterinary species [42]. An initial population of 190 clinically healthy dogs was recruited, exceeding recommended minimum of 120 animals to employ nonparametric statistical methods with 90% confidence intervals (CI) [42]. The initial population encompassed three distinct groups of animals: 82 privately-owned pet dogs, 56 working police dogs and 52 dogs residing in a rescue shelter. This aimed to represent the heterogeneity of the canine species. All animals were located in the Community of Madrid (Spain). Blood samples were collected during routine health checks at the Veterinary Teaching Hospital of the Complutense University of Madrid, between July 2021 and December 2022. These analyses included haematological and biochemical profiles, along with testing for antibodies against two prevalent vector borne pathogens in the

area (*Leishmania infantum* and *Ehrlichia canis)* [43, 44]. MDA and 8-OHdG concentration measurements were performed on the remaining serum volume from these routine health checks. Therefore, an ethics approval was not needed, as confirmed by the Veterinary Teaching Hospital criteria. Dog owners were informed of the use of remaining serum samples for scientific purposes.

Inclusion criteria for this study comprised dogs of any age, sex, breed, and size, demonstrating clinical health based on physical examination, including normal body condition, and laboratory results. Exclusion criteria included any history of pre-existing diseases, recent medication use (min. 1 month), or evidence of disease from the physical examination or laboratory analyses.

#### Blood sampling and preanalytical factors

Venous blood samples were collected and transferred into 0.5 mL tubes containing $K_3$ EDTA for the haematology profile. For biochemistry and serology analyses, blood was transferred into 5 mL plain tubes and centrifuged (1200 g, 10 min) for serum separation. The haematological and biochemical analyses were performed within 3 h of sample collection. Surplus serum samples were aliquoted and stored at -80ºC for cortisol, infectious diseases antibodies and OS biomarkers determination. OS biomarkers were determined in batches complying with their stability data [45, 46]. In view of the interference due to hemolysis, icterus and lipemia on TBARS assay, samples showing any of these alterations were excluded [47].

#### Laboratory analyses

The haematology profile was conducted on an automated haematology analyzer (URIT 2900Vet Plus TS®, URIT Global Diagnostics Supplier, China), which included red blood cell count (RBC), haemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cell count (WBC), platelet count (PLT) and mean platelet volume (MPV). Blood smears were stained with May-Grünwald-Giemsa, and evaluated for manual differential leukocyte counts [neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS) and basophils (BASO)]. The biochemistry profile was obtained using an automated biochemistry analyzer (TC220®, Jiangxi Tecom Science Corporation, China), which included 17 variables: urea (UREA), creatinine (CRE), total protein (TP), albumin (ALB), globulins (GLOB), albumin/globulins ratio (A/G), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALKP), lactate dehydrogenase (LDH), calcium (Ca), phosphate (P), calcium/phosphate ratio (Ca/P), total cholesterol (CHOL), uric acid (UA) and creatine kinase (CK). Serum cortisol was measured by a competitive ELISA, previously validated by the laboratory, as a potential indicator of psychogenic stress in the dogs [48–50]. In addition, serum antibodies against *Leishmania infantum* and *Ehrlichia canis* were determined by immunofluorescence antibody test (IFAT).

OS biomarkers were assessed using commercially available kits. Serum MDA concentration was determined using a TBARS assay kit (Cayman Chemicals, USA). This method relies on the reaction between MDA and thiobarbituric acid (TBA) under elevated temperatures and acidic conditions, producing an MDA-TBA adduct that is quantified spectrophotometrically at 540 nm. The results were expressed in MDA concentration (µM). This kit had been previously employed in canine serum and plasma samples [26, 36, 51].

Serum 8-OHdG was quantified by a competitive ELISA [*Highly Sensitive 8-OHdG Check*, *Japan Institute for the Control of Aging (Jaica)*, Japan], validated for canine samples by the manufacturer [46]. The assay included a filtration of serum samples prior to analysis using an ultra filter (cut off molecular weight 10,000) (Amicon® Ultra, Merck KGaA, Germany). Results were expressed in 8-OHdG concentration (ng/mL).

#### Statistical analysis

Statistical analysis for the establishment of RI was conducted following the ASVCP and the Clinical and Laboratory Standards Institute (CLSI) guidelines, using the set of macroinstructions for Microsoft Excel® *Reference Value Advisor* [42, 52, 53]. Descriptive analysis was provided and the normality of data distribution was assessed by means of histograms and the Anderson-Darling test, considering a $p$ value $< 0.050$ as statistically significant. The Tukey's test was employed to identify and remove potential outliers. Reference limits were then calculated through nonparametric methods, encompassing the central 95% of the reference values with 90% CI. The partitioning into the three study groups (pet dogs, police dogs and shelter dogs) was evaluated using the statistical criteria of Lahti et al. [54] and clinical considerations, as recommended [42, 55].

Univariate and multivariate statistical analysis were conducted to evaluate the influence of the sources of biological variation and the analytical variables on OS biomarkers. Firstly, univariate analysis was performed by non-parametric tests using SPSS® Statistics (IBM®, Spain). Differences between sexes were evaluated through the Mann-Whitney U test, and differences between variables with three or more categories [study groups and dog sizes (small, medium and large)] were assessed using the Kruskall-Wallis test with Bonferroni's correction. To examine the uniformity of groups in terms of dog sizes and sexes,

the demographic data of the groups were subjected to a Chi-squared test. The Spearman's Rho test was used to evaluate the statistical correlation between OS biomarkers and numerical variables (age, haematological, biochemical variables and serum cortisol). A $p$ value < 0.050 was considered statistically significant in every case.

Finally, to identify the factors that had a significant impact on OS biomarkers when evaluated as a whole, multivariate regression models were constructed for each OS biomarker using statistical software STATA™ (StataCorp LLC, USA). The OS biomarker (MDA or 8-OHdG) was defined as the dependent variable and the "reference dog" was defined as a male pet dog. The model evaluated the effect of the biological variables (group, sex, age, size), along with the effect of those numerical (haematological and biochemical) variables that had showed statistically significant association with each biomarker in univariate analysis.

## Results

### Reference population

Out of the initial population of 190 animals, 26 dogs were excluded from the study due to clinical data meeting exclusion criteria, or due to abnormal haematological or biochemical results. Additionally, 11 dogs were excluded due to positive serology tests, and 9 dogs were discarded in view of haemolytic or lipemic serum samples. As a result, statistical analysis was conducted on a reference population of 144 dogs.

The reference population ($n$ = 144) encompassed 57 pet dogs (40%), 43 police dogs (30%) and 44 shelter dogs (30%), of both sexes [76 males (53%) and 68 females (47%)], with ages ranging from 6 months to 16 years [mean 3,93 years and standard deviation (SD) 2,90], and various sizes [small ($n$ = 19; 13%), medium ($n$ = 28; 20%) and large ($n$ = 97; 67%)]. The population included 31 breeds: mixed-breed dogs (25% of the reference population), German Shepherd (14%), Belgian Malinois (14%), American Staffordshire Terrier (8%), Pitbull Terrier (7%), Labrador Retriever (5%), Spanish Greyhound (3%), Podenco (3%), and others (see supplementary material).

Pet dogs group ($n$ = 57) consisted of 29 males (51%) and 28 females (49%), ranging in age between 6 months and 10 years (mean 3.21; SD 2.81), sized small (28%), medium (37%) and large (35%). Pet dogs belonged to multiple breeds, mainly mixed-breed dogs (32%), Labrador Retriever (7%), Podenco (7%), Maltese (5%) and others. The group of police dogs ($n$ = 43) consisted of 18 males (42%) and 25 females (58%), between 6 months and 9.5 years of age (mean 4.83; SD 2.37). All police dogs were large, being Belgian Malinois (47%), German Shepherd (44%), Labrador Retriever (5%), German Shorthaired Pointer (2%) and mixed-breed dogs (2%). Shelter dogs group ($n$ = 44) included 29 males (66%), 15 females (34%), ranging from 6 months to 16 years of age (mean 3.98; SD 3.28), of the three sizes [small (7%), medium (16%) and large (77%)]. Shelter dogs belonged to various breeds [mixed-breed (39%), American Staffordshire Terrier (25%), Pitbull Terrier (21%) Spanish Greyhound (7%) and others] (see supplementary material).

The Chi-squared test showed no significant difference in sex distribution between the groups ($p$ = 0.075). Conversely, the groups were not uniform in dog sizes ($p$ < 0.001).

### Reference intervals

Descriptive statistics and the RI established for serum MDA and 8-OHdG, obtained with nonparametric methods in *Reference Value Advisor*, are presented in Table 1. The frequency histograms of both biomarkers are illustrated in Figs. 1 and 2. The Anderson-Darling method revealed both biomarkers followed non-Gaussian distributions ($p$ < 0.001). The Tukey's test identified one outlier in the distribution of 8-OHdG, which was deemed an aberrant observation and subsequently excluded from the reference population. Conversely, other observations flagged as "suspicious" by the Tukey's test for MDA and 8-OHdG were retained in the absence of evidence of disease or analytical inaccuracies. As a result, the final reference population comprised 144 dogs for MDA and 143 dogs for 8-OHdG.

Mean MDA concentrations in the three study groups were 8.14 µM (SD 3.03) in pet dogs; 7.30 µM (SD 3.32) in police dogs; and 6.16 µM (SD 3.31) in shelter dogs. Mean 8-OHdG concentrations were 0.43 ng/mL (SD 0.21) for pet dogs; 0.41 ng/mL (SD 0.19) for police dogs; and 0.43 ng/mL (SD 0.19) for shelter dogs. According to Lahti et al. (2004) statistical recommendations for non-Gaussian distributions [54], partitioning into the three study groups (pet, police, and shelter dogs) could be applied for both biomarkers. However, statistical results from multivariate analysis and clinical considerations were also taken into account, as recommended [42, 54, 55], which did not support partitioning. Therefore, the entire population RI were retained for both biomarkers.

### Univariate analysis

The Kruskal-Wallis test showed statistically significant differences between groups for MDA ($p$ = 0.006), but not for 8-OHdG ($p$ = 0.835) (Figs. 3 and 4). The concentration of MDA was found to be significantly lower in shelter dogs compared to pet dogs ($p$ = 0.004), but no statistically significant differences were observed between the other groups. No significant differences between sizes were found by the Kruskal-Wallis test for MDA ($p$ = 0.196), nor 8-OHdG ($p$ = 0.605). Similarly, no significant differences between sexes were found by the Mann-Whitney U test for MDA ($p$ = 0.090) nor 8-OHdG ($p$ = 0.733).

**Table 1** Reference intervals (RI) for serum malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the canine population of study. The Anderson-Darling test was used for distribution's evaluation ($p < 0.050$). N, number of valid observations; SD, standard deviation; NG, non-Gaussian; NP, nonparametric; LRL, lower reference limit; URL, upper reference limit; CI, confidence interval

| Measurand | Units | *n* | Mean | SD | Median | Min | Max | *p*-value | Distribution | Method | LRL of RI | URL of RI | CI 90% of LRL | CI 90% of URL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MDA | µM | 144 | 7,28 | 3,3 | 7,08 | 0,17 | 18,75 | 0,005 | NG | NP | 1,85 | 14,51 | 0,17 – 2,36 | 13,19 – 18,75 |
| 8-OHdG | ng/mL | 143 | 0,42 | 0,2 | 0,46 | 0,05 | 0,84 | 0,000 | NG | NP | 0,06 | 0,75 | 0,05 – 0,09 | 0,71 – 0,84 |

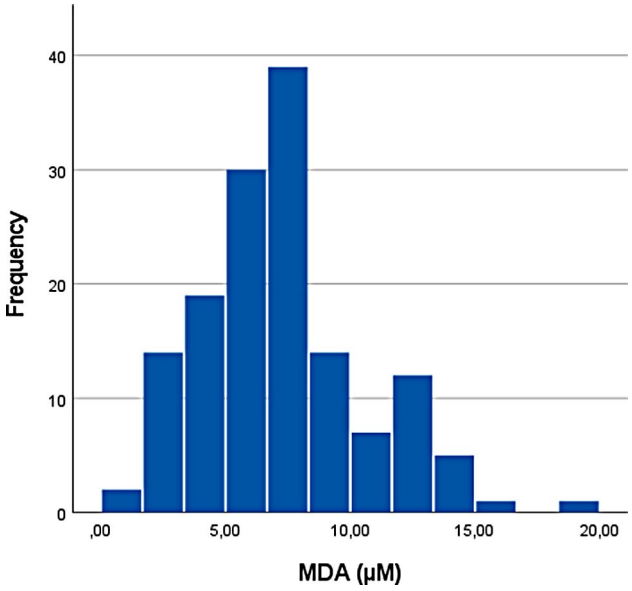


**Fig. 1** Histogram depicting the frequency distribution for serum malondialdehyde (MDA) in the reference population ($n = 144$). Serum MDA distribution was non-Gaussian, according to the Anderson-Darling test ($p < 0.001$)

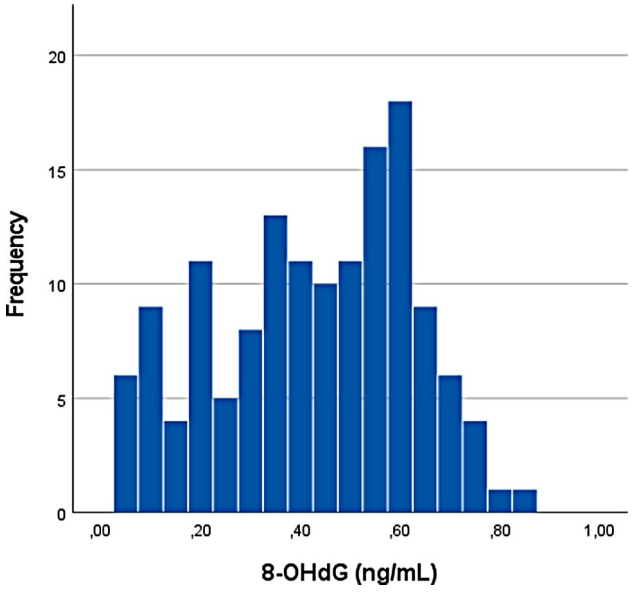


**Fig. 2** Histogram depicting the frequency distribution for serum 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the reference population ($n = 143$). Serum 8-OHdG distribution was non-Gaussian, according to the Anderson-Darling test ($p < 0.001$)

The Spearman's Rho method revealed no significant correlation between age and MDA ($p = 0.681$) nor 8-OHdG ($p = 0.203$). Regarding analytical variables, MDA showed significant correlation coefficients (R) with MONO ($p = 0.027$, $R$=-0.185), EOS ($p = 0.048$, $R$=-0.165) and CHOL ($p < 0.001$, $R = 0.518$). 8-OHdG showed significant correlations with UREA ($p < 0.001$, $R = 0.402$), CRE ($p = 0.007$, $R = 0.229$) and AU ($p = 0.008$, $R = 0.225$).

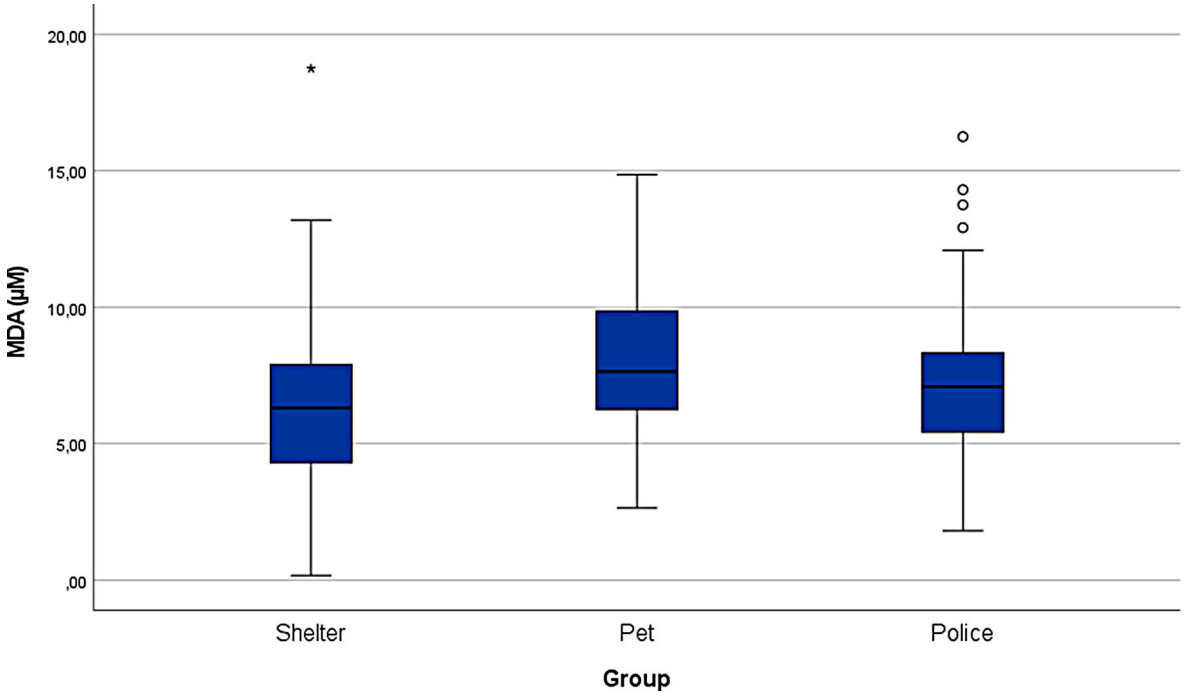


**Fig. 3** Box plots showing median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers), of serum malondialdehyde (MDA) results in the three groups of study (pet, police and shelter dogs). Data points lying between 1.5 (○) and 3 times (*) the interquartile range above the third quartile or below the first quartile are represented in the figure

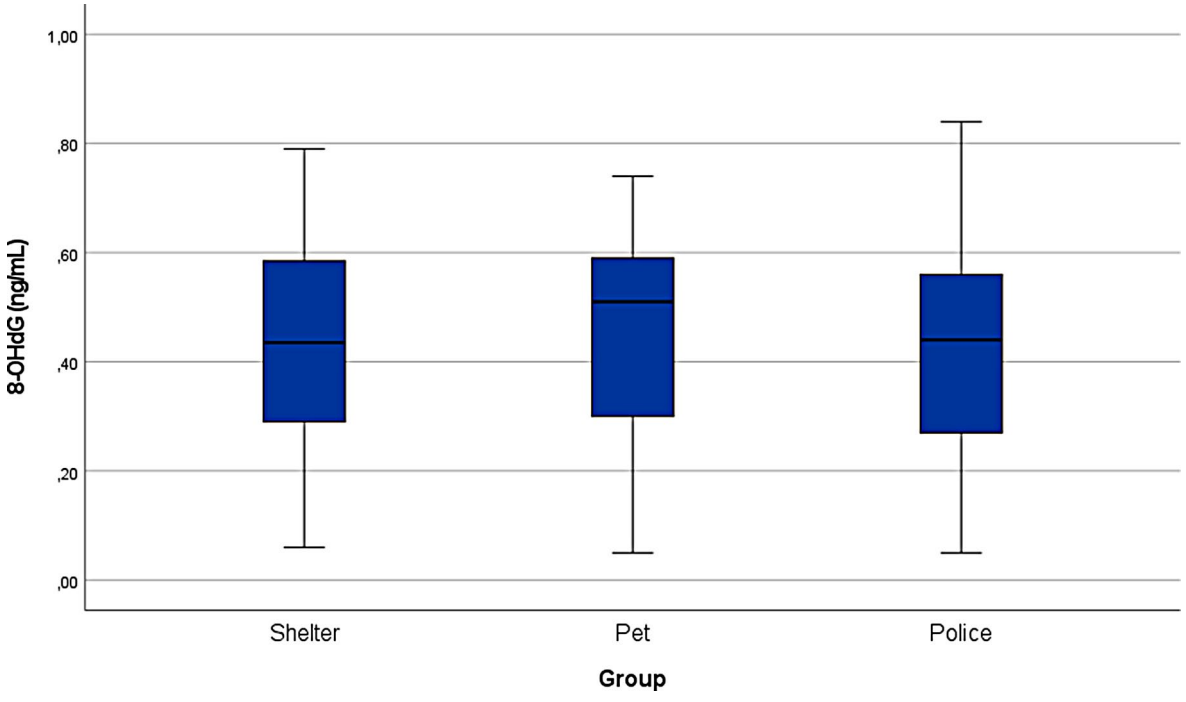


**Fig. 4** Box plots showing median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers), of serum 8-hydroxy-2′-deoxyguanosine (8-OHdG) results in the three groups of study

**Table 2** Output of the final multivariate regression model for factors associated with serum malondialdehyde (MDA) in the canine reference population. Statistically significant associations ($p < 0.050$) are displayed in bold. SE, standard error; CI, confident intervals; MONO, monocytes; CHOL, cholesterol; 8-OHdG, 8-hydroxy-2′-deoxyguanosine

| MDA | Coefficient | SE | t value | *p* value | 95% CI | |
|---|---|---|---|---|---|---|
| Group | | | | | | |
| Police | -0.4642142 | 0.5353112 | -0.87 | 0.387 | -1.522966 | 0.594538 |
| Shelter | **-1.401647** | 0.5326891 | -2.63 | **0.010** | -2.455214 | -0.3480812 |
| MONO | **-1.453809** | 0.6846023 | -2.12 | **0.036** | -2.807833 | -0.0997851 |
| CHOL | **0.0328542** | 0.0043803 | 7.50 | **0.000** | 0.0241907 | 0.0415176 |
| 8-OHdG | **3.999976** | 1.149238 | 3.48 | **0.001** | 1.726984 | 6.272967 |
| Constant | 0.2718914 | 1.152349 | 0.24 | 0.814 | -2.007254 | 2.551037 |

**Table 3** Output of the final multivariate regression model for factors associated with serum 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the canine reference population. Statistically significant associations ($p < 0.050$) are displayed in bold. SE, standard error; CI, confident intervals; MDA, malondialdehyde

| 8-OHdG | Coefficient | SE | t value | *p* value | 95% CI | |
|---|---|---|---|---|---|---|
| UREA | **0.0064659** | 0.0013167 | 4.91 | **0.000** | 0.0038623 | 0.0090696 |
| MDA | **0.0117486** | 0.0045499 | 2.58 | **0.011** | 0.0027514 | 0.0207458 |
| Constant | 0.0790695 | 0.0609389 | 1.30 | 0.197 | -0.0414329 | 0.1995719 |

Spearman's Rho test also revealed a significant correlation between MDA and 8-OHdG values ($p = 0.008$, $R = 0.219$). The rest of the analytical variables, including serum cortisol, did not show significant correlations with OS biomarkers.

### Multivariate regression models

To evaluate the influence of the biological factors (group, sex, size and age) along with the analytical variables that showed significant correlation with each biomarker in the univariate analysis, a multivariate regression model was constructed for each biomarker. The model for MDA revealed significant positive influences of CHOL ($p < 0.001$) and 8-OHdG ($p = 0.001$) values, and a significant negative influence of MONO value ($p = 0.036$). Additionally, shelter dogs were found to display significantly lower MDA values than pet dogs ($p = 0.010$). The rest of the biological (sex, age and size) and analytical variables (EOS) did not significantly impact MDA value. The model for 8-OHdG confirmed the positive relationship between 8-OHdG and MDA ($p = 0.011$), and the positive influence of UREA ($p < 0.001$). The associations between 8-OHdG and CREA or AU were discarded by the model. Coefficient estimates, standard errors, and *p*-values obtained for each variable are displayed in Tables 2 and 3.

## Discussion

Even though MDA and 8-OHdG have been suggested as clinically valuable biomarkers in multiple canine diseases and conditions [9, 17–40], their application in clinical settings is still scarce, likely due to the absence of established RI and the discrepancies between analytical techniques [6, 7, 11, 41]. The present study is the first to report assay-specific RI for MDA and 8-OHdG in the canine species, as well as identifying various significantly related haematological and biochemical factors.

### RI for serum MDA and 8-OHdG

RI for both biomarkers were obtained through *Reference Value Advisor*, calculating reference limits with 90% CI using nonparametric methods, and encompassing the central 95% of the observations. Normality of distributions were assessed using the Anderson-Darling test, and outliers were identified using the Tukey's test [53].

Serum MDA and 8-OHdG concentrations in the reference population ($n = 144$ and $n = 143$, respectively) were found to follow non-Gaussian distributions. In contrast to some thoroughly regulated biochemical analytes (such as electrolytes or glucose), other biochemical variables do not typically follow Gaussian distributions [56], which could apply to these by-products of oxidative damage, influenced by the multiple factors affecting redox homeostasis [4].

The present study established a RI for canine serum MDA concentration of 1.85 to 14.51 µM (mean 7.28; SD 3.29), being the first RI reported for this analyte in a large population, as recommended in the ASVCP guidelines [42], and serving as an stepping stone for its utilization in clinical settings. The only previous study that suggested RI for plasma MDA in dogs obtained lower results, but comprised a rather limited population in number and biological sources of variation (56 medium-sized mongrel dogs, aged 3–4 years) [41]. In accordance with our results, various studies have reported blood MDA concentrations in control dogs within our RI [31, 36]. Other studies obtained higher or lower plasma/serum MDA values in small groups of healthy dogs [17, 23, 51], which

could be attributed to the analytical variations of TBARS assay [6, 7, 11].

This study established a RI for canine serum 8-OHdG concentration of 0.06 to 0.75 ng/mL (mean 0.42; SD 0.20). While previous works have measured 8-OHdG in canine tissues and other body fluids [38, 39, 57–59], this is the first study reporting a canine serum RI. This finding holds potential for expanding the investigation of DNA oxidative damage in canine pathology through the use of serum samples. Our finding could only be compared to a single study reporting a serum mean value of 1.44 ng/mL in a control canine population, although the antibody used in their assay recognizes other damaged nucleic species besides 8-OHdG, yielding higher values [37]. Other studies have measured plasma or serum 8-OHdG in dogs [40, 60, 61], but lacked necessary data (numerical results and pre-filtration details) for a proper comparison.

For both MDA and 8-OHdG, it was decided to preserve the entire reference population RI rather than partitioning into study groups (pet, police and shelter dogs) in the absence of clinical criteria or published data that supported otherwise [42, 54, 55].

#### Influence of biological factors on OS biomarkers

While the univariate analysis identified various potential influences, the multivariate models provided a more comprehensive approach, revealing the biological and analytical factors significantly associated with MDA and 8-OHdG. Regarding the sources of biological variation, neither MDA nor 8-OHdG showed significant associations with age or size of the animals. Although OS has been associated with cellular senescence, the relationship between age and oxidation rate has not been proven in all animal models, and seems to be non-linear, probably due to other affecting factors such as genotype, gene expression and mitochondrial function [62]. Additionally, as a result of the correlation between size and lifespan in dogs, heterogeneous populations such as the present one may fail to reveal associations between OS biomarkers and age or size separately [57, 63–65]. This study did not find significant associations between sex and MDA or 8-OHdG. While a prior study reported variations in ROS levels across the oestrus cycle in female dogs [66], information on the reproductive status was not recorded in our study, limiting further exploration of this aspect. Additionally, the breed factor could not be assessed due to the racial heterogeneity of the reference population.

We measured serum cortisol to assess psychogenic stress aiming to investigate if police or shelter dogs experienced heightened psychogenic stress and its potential relationship with OS biomarkers. However, no significant associations were found between cortisol and MDA nor 8-OHdG. This could be attributed to the inherent difficulties in assessing of psychogenic stress through analytical variables, and the documented lack of specificity of glucocorticoids in this aspect [67–73]. Lastly, the regression model showed lower MDA in shelter dogs. No clear clinical explanations were found for this finding, which could be influenced by an individual lower value (0.17 μM) that was not flagged as *outlier* by the software but may have influenced the model.

#### Associations between OS biomarkers and haematological and biochemical variables

The regression model for serum MDA revealed positive associations with 8-OHdG and CHOL values, and a negative association with MONO. The positive relationship between MDA and 8-OHdG concentrations would be fully in line with the underlying mechanisms of OS. Firstly, it seems reasonable that those individuals that are more exposed to OS experienced increased lipid and DNA oxidation, especially considering that many reactive species, such as hydroxyl radicals (•OH), are able to oxidize both biomolecules [74–76]. Secondly, it would be plausible that increased lipid peroxidation induced enhanced DNA oxidation. This would be supported by the fact that oxidized membrane phospholipids experience structural damages that affect cellular integrity [77–79], which may increase the exposure of nucleic acids to oxidation. Furthermore, oxidation of fatty acids produces lipid peroxides and other aldehydes, such as MDA, acrolein or isoprostanes, that are chemically active and capable of diffusing throughout the cytoplasm and further oxidizing other molecules, namely proteins and nucleic acids [11, 16, 80–82]. A previous study reported increased levels of both MDA and 8-OHdG in mammary tissue of dogs with carcinomas [39], but the present work would be the first to find a significant association between both biomarkers in canine serum.

The positive association between MDA and CHOL values could be explained by in vivo and in vitro mechanisms. Increased CHOL levels have been found in dogs with higher body condition scores [83], and a rise in lipid peroxidation, as demonstrated by higher MDA values, has been reported in obese dogs [26] and dogs with hyperlipidemia [27]. The present study encompassed healthy dogs and excluded underweight and overweight dogs, but it is possible that individuals with a higher body fat percentage presented an increase in tissue lipid peroxidation. Furthermore, along with membrane phospholipids, CHOL itself is one of the main targets of lipid peroxidation by free radicals and other oxidants, potentially yielding several products such as MDA [84]. This could represent another in vivo mechanism of this relationship. In vitro explanations could be related to the ability of TBA to react with other lipids, apart from MDA, during TBARS assay [7, 8, 11, 85]. In this study, visually lipemic samples were excluded in light of the

interference of lipemia with the assay [47]. However, given that circulating CHOL is transported by various lipoproteins [56], it seems possible that samples with higher CHOL levels also carried other lipids capable of cross-reacting with TBARS assay.

The model also showed an inverse association between MDA and MONO values. It has been reported that MDA forms adducts with low-density lipoproteins (LDL) (MDA-LDL), which are recognized by macrophages' *scavenger receptors*, leading to their internalization and foam cell formation [86]. Moreover, MDA-LDL adducts seem to trigger macrophage apoptosis, mediated by immune complexes (IC) and tumour necrosis factor (TNF) [87]. While investigating these mechanisms would require more complex analytical methods, it could be plausible that similar processes could contribute to the observed inverse relationship between MDA and MONO values.

The regression model for 8-OHdG confirmed its positive association with MDA concentration, and revealed a positive association with UREA. The latter could be attributed to the renal excretion of both analytes [88]. Notably, 8-OHdG has been reported as a valuable biomarker in humans with chronic renal disease [88–90], renal carcinoma [91], and diabetic nephropathy [92] but, to our knowledge, this would be the first study to find associations between serum UREA and 8-OHdG concentrations in dogs.

Limitations of the present study include the lack of information regarding the dogs' diet and reproductive status. Further studies are needed to investigate the potential influence of these factors on MDA and 8-OHdG values.

## Conclusions

OS has been proven to be a critical mechanism in the pathogenesis of several canine diseases, but the data on RI and biological and analytical factors associated with two of the most widely used biomarkers is still scarce. The present study is the first to establish assay-specific RI for serum MDA (1.85–14.51 µM) and 8-OHdG (0.06–0.75 ng/mL) in a large and heterogeneous population of dogs. Additionally, the study revealed a positive relationship between both biomarkers, in line with the biochemical basis of OS, and demonstrated significant associations between MDA, CHOL and MONO values; and 8-OHdG and UREA concentrations. These data seem to have promising clinical value and could favour the application of MDA and 8-OHdG in a broader clinical spectrum.

### Abbreviations

| | |
|---|---|
| 8-OHdG | 8-hydroxy-2'-deoxyguanosine |
| A/G | Albumin/globulins ratio |
| ACVCP | American Society for Veterinary Clinical Pathology |
| ALB | Albumin |
| ALKP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| AST | Aspartate aminotransferase |
| BASO | Basophils |
| Ca/P | Calcium/phosphate ratio |
| Ca | Calcium |
| CHOL | Total cholesterol |
| CK | Creatine kinase |
| CLSI | Clinical and Laboratory Standards Institute |
| CRE | Creatinine |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EOS | Eosinophils |
| GGT | Gamma-glutamyltransferase |
| GLOB | Globulins |
| HB | Haemoglobin |
| HCT | Hematocrit |
| IFAT | Immunofluorescence antibody test |
| LDH | Lactate dehydrogenase |
| LDL | Low-density lipoproteins |
| LYM | Lymphocytes |
| MCH | Mean corpuscular haemoglobin |
| MCHC | Mean corpuscular haemoglobin concentration |
| MCV | Mean corpuscular volume (MCV) |
| MDA | Malondialdehyde |
| MONO | Monocytes |
| MPV | Mean platelet volume |
| NEU | Neutrophils |
| OS | Oxidative stress |
| P | Phosphate |
| PLT | Platelet count |
| RBC | Red blood cell count |
| RI | Reference intervals |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| TBA | Thiobarbituric acid |
| TBARS | Thiobarbituric Acid Reactive Substances |
| TNF | Tumour necrosis factor |
| TP | Total protein |
| UA | Uric acid |
| UREA | Urea |
| WBC | White blood cell count |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04614-1.

Supplementary Material 1

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### Author contributions

Perez-Montero B.: Investigation, conceptualization, performing analytical methodology, data curation and writing original draft. Fermin-Rodriguez ML.: Conceptualization, supervision and reviewing original draft. Portero-Fuentes M.: Sample collection, reviewing original draft. Sarquis J.: IFAT methodology application, reviewing original draft. Caceres S.: Serum cortisol quantification, reviewing original drats. Illera del Portal J.C.: Serum cortisol quantification, providing resources. De Juan L.: Project administration, providing resources and reviewing original draft. Miro G.: Project administration, supervision and reviewing original draft. Cruz-Lopez F.: Supervision, data curation, validation of results, writing, reviewing and editing original draft.

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**Data availability**
The data and materials are available from the corresponding author upon reasonable request.

## Declarations

**Ethics approval and consent to participate**
The authors confirm that the ethical policies of the journal, described on the journal's author guidelines page, have been adhered to. No ethical approval was required given that this study was performed with serum samples drawn for routine analysis. Dog owners were informed of the use of remaining serum samples for scientific purposes.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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## Publisher's note