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Study on the expression of testin in the testes of dogs

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Abstract

Introduction: Testin is a protein involved in cell mobility, adhesion and colony formation. In rats, testin presence has been reported in the testes, and its possible role in spermatogenesis has been suggested. Studies in humans also suggest a possible role of testin as a cancer suppressor protein. In the dog, which represents both an important pet species and a good animal model for studying biological and pathological testicular processes, the presence of testin has never been reported. **Material and Methods:** In the present study, the expression of testin in foetal, prepubertal, adult and aged canine testes was investigated. Testes from 5 adult and 3 aged dogs, from 2 one-month-old puppies and from 2 foetuses miscarried at the end of pregnancy were immunohistochemically examined with a commercial antibody against testin. **Results:** Testin was intensely expressed in Sertoli cells in every testis examined. Spermatids were also positive for testin in mature dogs and in the testicular areas of the aged ones which were not atrophic. Weak expression of testin was also detected in all testes examined. **Conclusion:** The present study, the first demonstrating the presence of testin in canine testes, provides the basis for further dog–human comparative research and for studies on the role of this protein in canine physiology, reproduction and testicular pathologies.

Keywords: testin, testes, dog, immunohistochemistry, TES gene.

Introduction

Testin, initially purified from Sertoli cell–enriched culture medium (5, 6, 12), is a protein with a molecular mass of 47 kDa, encoded by the TES gene located on the fragile site FRA7G at 7q31.2 (25, 30). The secretion of testin seems to be influenced by two interacting membrane proteins of 28 and 45 kDa (5, 6, 13, 14). The protein is composed of N-terminal PET (Prickle, Espinas, TES) and three C-terminal LIM (lin-11, isl-1, mec-3) domains at the COOH terminus, one LIM domain containing a loosely conserved cysteine-rich consensus sequence including two separate F1 zinc fingers. They are separated from each other by a spacer region (25, 34). Proteins of the LIM family have been found to be a part of the cytoskeleton (20, 25). They are responsible for protein–protein interactions coordinating signalling of intracellular pathways (25, 30, 32). Testin is localised along the actin stress fibres, at the cell–cell junction, and at foci of adhesion (11), and may interact with other cytoskeletal proteins, playing a significant role in cell adhesion, motility, and the reorganisation of the actin cytoskeleton (7, 19, 21, 25, 31). Interaction between the LIM3 domain of testin and Mena (mammalian-enabled) protein, which is an actin-regulatory protein and a modulator of cellular migration and adhesion (18, 19), has also been recently highlighted (7, 25).

Considering testin localisation, studies performed on rats revealed that this protein is present in all tissues, but mostly in the testes and ovaries (6, 13, 15). In the testes, in particular, testin has been demonstrated in rat Sertoli cells (SCs) to be a component of the tight junction which links these cells that is remodelled when

the developing germ cells pass from the basal to the adluminal compartment of seminiferous tubules (2, 9, 15, 24). The higher expression of testin in gonads, compared with other tissues, suggests that its expression may be related to the constant turnover of the junctions during germ cell and follicle development (15). Moreover, molecular studies characterised testin as a tumour-suppressor gene and reported its downregulation in several human malignancies (8, 16, 17, 22, 25).

In human medicine, wide panels of immunohistochemical markers are commonly used in pathology to study neoplastic and non-neoplastic diseases. Most of these markers have also been tested in canine tissues, the dog being considered a reliable comparative model for the study of various human biological processes and pathologies (1, 3, 4, 11, 29). These include gonadal disorders and testicular tumours, the frequency of which is increasing in the human species and which represent one of the most common spontaneous neoplasms in dogs (11, 33). Moreover, since humans and dogs share similar environmental conditions, scientists are constantly looking for new markers and proteins that will enable understanding of biological and pathological processes not only in humans but also in canine species.

Testin represents an interesting protein to investigate in comparative testicular pathology. However, before any study related to the expression of a date marker is conducted in pathological tissues, the marker must be investigated in normal and (if possible) in developing ones. Therefore, the aim of the present study was to investigate the presence and the immunohistochemical physiological expression of testin in foetal, prepubertal, adult and aged canine testes.

Material and Methods

Paraffin blocks from 12 canine testes were retrieved from the archive of the Department of Pathology at Wrocław University of Environmental and Life Sciences. Five testes without any pathological changes and removed in routine castration were from adult dogs (2–6 years old). Three testes with areas of atrophy of the seminal epithelium and also removed in routine castration were from aged dogs (9 or 10 years old), two testes were from month-old puppies which died of traumatic injuries and two were from foetuses miscarried at the end of pregnancy. No clinical fertility information was available for the adult or aged dogs.

Histology. All samples were fixed for 24 h in 10% buffered formalin, and from all of them a complete longitudinal section, including testicular parenchyma and epididymis, was obtained and routinely processed for histology by dehydration in graded alcohols, clarification in xylene and embedding in paraffin. Serial three-micrometre-thick sections were cut from the paraffin blocks and mounted on Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany). One section was stained with haematoxylin and eosin while other sections were immunohistochemically examined.

Immunohistochemistry. Immunohistochemical tests were performed on a Leica BOND-MAX (Leica Biosystems, Deer Park, IL, USA) according to the following protocol. First, tissues were deparaffinised with BOND Dewax Solution (Leica Biosystems) and pre-treated with BOND Epitope Retrieval Solution 1 (Leica Biosystems) for 20 min. The activity of the endogenous peroxidase was blocked by Peroxidase Block using the BOND Polymer Refine Detection System (Leica Biosystems). Testin antibody (NBP1-87987; Novus Biologicals, Centennial, CO, USA), diluted 1:100 in BOND Primary Antibody Diluent (Leica Biosystems), was applied as the primary antibody for 15 min at room temperature. Next, the samples were incubated with Post Primary Block and Polymer (Leica Biosystems) using the BOND Polymer Refine Detection System. The substrate for the reaction was 3,3′-diaminobenzidine chromogen. The sections were then counterstained with haematoxylin in the BOND Polymer Refine Detection System. A negative control was obtained by replacing the primary antibody with BOND Primary Antibody Diluent. Rabbit serum was used as a negative control for staining specificity at the same protein concentration as the primary antibody in place of the primary antibody. Immunohistochemical results were assessed using a modified semiquantitative immunoreactive score on the scale according to Remmele and Stegner (28) reported in Table 1.

Table 1. Immunoreactive scores on the semiquantitative scale established by Remmele and Stegner (28)

Percentage of positive cells	Points	Immunohistochemical reaction	Points
0	0	Absent	0
1–10%	1	Weak	1
11–50%	2	Moderate	2
5–80%	3	Intense	3
>80%	4		

Immunoblotting. Canine tissue from three testes were homogenised in 500 µL of ice-cold Tissue Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA) with 50 µL of Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA). After incubation of the homogenates on ice for 30 min and centrifugation at 10,000 × *g* at 4°C, the homogenates' protein content was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Protein samples of 100 µg were resolved in reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis at 12% gel strength and 95°C for 10 min and transferred onto a polyvinylidene fluoride membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The membrane was treated with Pierce Western Blot Signal Enhancer (Thermo Fisher Scientific), blocked for 1 h with 5% nonfat milk in phosphate-buffered saline (PBS) containing 0.5% (v/v) Triton X-100 (Sigma-Aldrich) and incubated overnight at 4°C with the Testin antibody used in immunohistochemistry diluted 1:250. Primary antibodies were detected with an anti-IgG HRP-conjugated antibody, and blots were developed using a SuperSignal West Femto enhanced chemiluminescence substrate (Thermo Fisher Scientific). The relative intensities of the bands were determined using Quantity One software (Bio-Rad Laboratories). Antibody dilution buffer (5% nonfat milk in PBS containing 0.5% (v/v) Triton X-100) and the rabbit serum at the same protein concentration as the primary antibody were used in place of the primary antibody solution as negative (secondary only) controls.

Testis sections underwent microscopy with computer aided image analysis using an Olympus BX53 optical microscope (Olympus, Tokyo, Japan) equipped with a digital Olympus ColorView IIIu camera. The measurements were taken using cellSens software (Olympus Soft Imaging Solutions, Münster, Germany).

Protein homology detection. In order to confirm the homology of canine and human testin protein, the Basic Local Alignment Search Tool (National Library of Medicine, National Institute of Health, Bethesda, MD, USA) was used.

Results

Histology. In the testes from the five adult dogs in the 2–6 year age range, a complete seminal line including spermatozoa was recognisable in the seminiferous tubules. In the aged dogs, which were 9 or 10 years old, areas of normal seminiferous tubules and areas presenting tubular atrophy coexisted. In these latter areas, spermatogenesis was frequently arrested at early stages of maturation. Tubules lined only by Sertoli cells were also present, scattered or arranged in groups. In the testes of the two puppies, spermatogonia were recognisable, while in both foetuses, the seminiferous tubules were very small, had no recognisable lumen, and were lined by undifferentiated SCs and rare early germ cells.

Immunohistochemistry. In the testes of the adult dogs, intense cytoplasmic testin expression was observed in SCs, spermatocytes and spermatids. In the interstitium, interstitial endocrine Leydig cells were more weakly positive for testin (Fig. 1A and B). In the testes of older dogs, normal areas reacted as in younger adult dogs, while in atrophic areas, a very weak cytoplasmic reaction was observed exclusively in SCs (Fig. 1C). The testes of the puppies showed weaker expression of testin compared to normal adult canine organs in SCs as well as in interstitial endocrine Leydig cells (Fig. 1D). In the testes of miscarried foetuses, a positive but weak cytoplasmic reaction was only observed in the population of SCs (Fig. 1E, Table 2).

Immunoblotting. Western blot analysis was used to characterise the expression pattern of TES in normal mature canine testes. In the tested tissue, a clear dark band was obtained between 55 kDa and 40 kDa which was not obtained in the standard. The mass of the visible band was approximately 47 kDa, and therefore it represented the testin protein, while the other bands observed in Fig. 2 may represent possible degradation products of TES. In addition, the approximately 42 kDa band that was visible in all samples could be an isoform of TES (17).

Table 2. Immunohistochemical results

	Germ cells/Spermatocytes		Sertoli cells		Leydig cells	
	% of positive cells	Reaction intensity	% of positive cells	Reaction intensity	% of positive cells	Reaction intensity
Normal mature testes	4	3	4	3	3	2
Atrophic areas of aged canine testes	0	0	4	1	0	0
Normal immature testes	0	0	4	1	3	1
Testes of miscarried foetuses	0	0	2	1	0	0

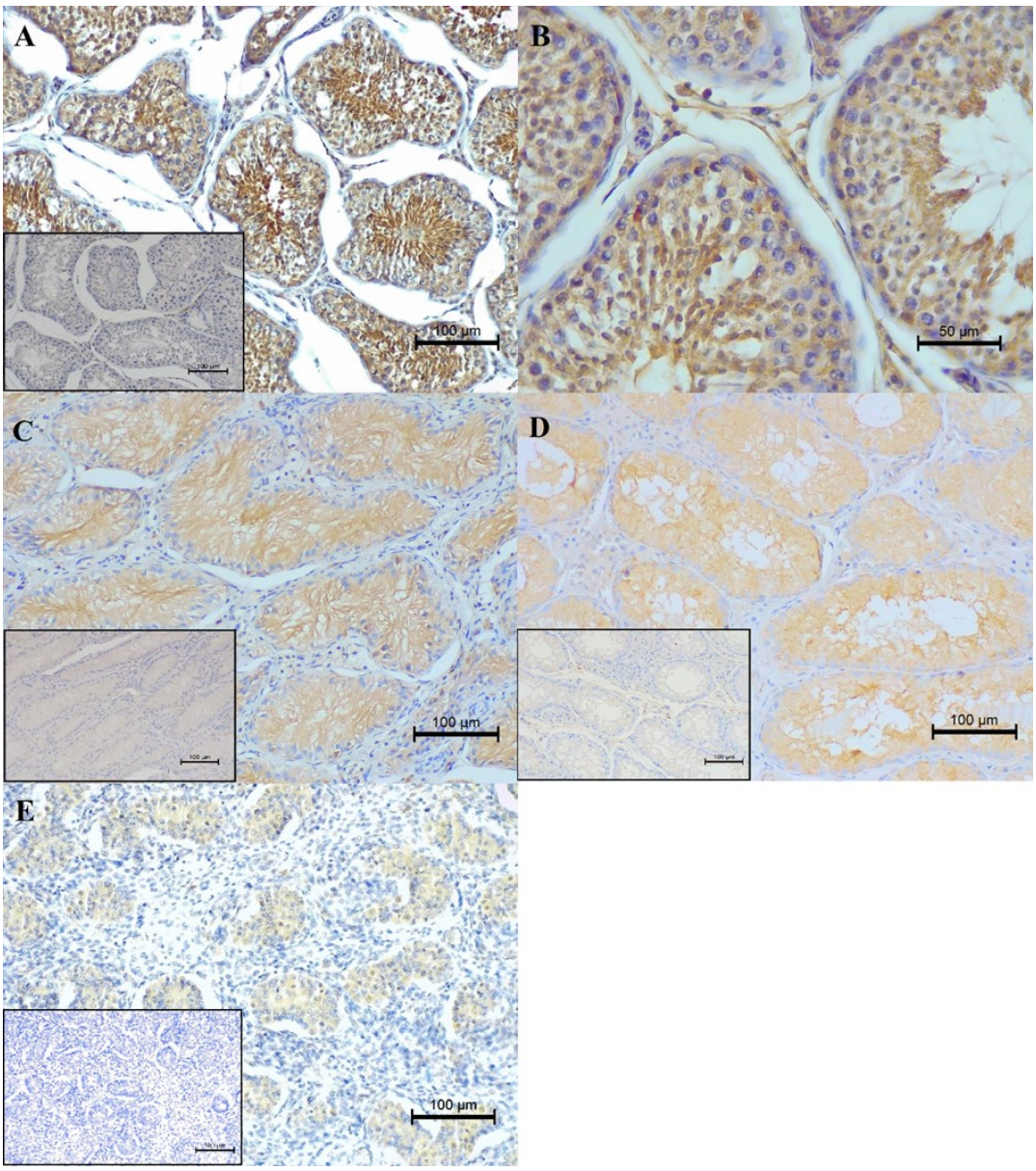

Fig. 1. Immunohistochemical examination of the expression of testin in the testes of dogs at various life stages. (A) Intense cytoplasmic testin expression in the Sertoli cells (SCs) and spermatids of an adult dog aged between 2 and 6 and weaker expression in the interstitium and interstitial endocrine Leydig cells. The lower left corner shows the negative control. (B) The same cells at higher magnification. (C) Very weak cytoplasmic reaction exclusively in the SCs of an older dog aged 9 or 10. The left corner shows the negative control. (D) Weaker expression of testin compared to normal adult canine testes in the SCs and interstitial endocrine Leydig cells of a puppy aged 2 months. The lower left corner shows the negative control. (E) Positive but weak cytoplasmic expression in the SCs of a miscarried canine foetus. The lower left corner shows the negative control. (A) and (C–E) scale bar 100 µm; (B) scale bar 50 µm

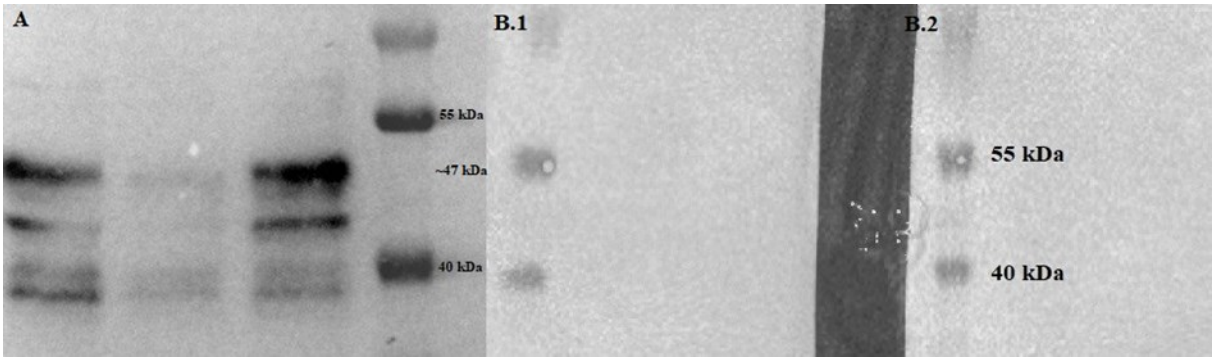

Fig. 2. Representative Western blot results of TES protein expression in testes. (A) A marked dark band between 55 kDa and 40 kDa corresponds to a testin protein of ~47 kDa. (B.1) Negative control without primary antibody. (B.2) Negative control with rabbit serum

Protein homology detection. The result confirmed homology at the level of 97% between human and canine protein: identities: 400/412 (97%), positives: 403/412 (97%), gaps: 0/412 (0%). The demonstrated high homology (at the level of 97%) is proof that the antibody against human testin protein may also be adapted for use in dogs (human protein length: testin isoform 2 (*Homo sapiens*) query ID: NP_690042.1 length: 412. Canine protein length: (*Canis lupus familiaris*). Sequence ID: NP_001162159.1 length: 421).

Discussion

Testin is a protein that is localised in the cytoplasm of cells in both normal and cancerous tissues, with a role in biological processes in humans which is still being explored. It has not yet been fully understood and described in dogs, and has only been identified and described in the rat (5, 6, 12, 14) to the best of our knowledge. The protein's expression in other species has also not yet been investigated.

In the studies in rats, it was demonstrated that testin is a protein secreted by SCs which may be closely bound to the surface of the cell. It was also noticed that the level of testin expression varies depending on the phase of the spermatogenesis cycle, and that protein expression is especially localised at the point of contact of the reproductive epithelium with SCs (13, 35). Grima *et al.* (12) showed that testin can become tightly associated with the testicular cell membrane to the extent that solubilisation from the Sertoli or testicular membrane requires the use of detergents. In addition, both the SC testin secretory activity and the mRNA level are tightly coupled to the integrity of specialised junctions between testicular cells. Moreover, the maintenance of steady-state testin mRNA is also correlated with an extensive renewal of cell–cell junctions during development, suggesting that testin is a marker in monitoring germ cell–Sertoli cell interactions throughout spermatogenesis (12).

The full role of testin in the animal body may not have been demonstrated yet because research in other animals than the rat is still awaited. The far longer lifespan of a dog than of a rat lengthens the impact of various factors of the environment in which an owner and a dog live. Therefore, it seems that the dog may be a better research model for the role of the testin protein, not only in physiological but also in oncological processes in the bodies of animals and humans.

Considering our results, the demonstration of the presence of testin in normal mature canine testes by the Western blot method indicated the presence of testin in this species and confirmed the cross-reaction of the antibody employed with the canine protein, corroborating the immunohistochemical results obtained. Western blot is usually employed in veterinary medicine to confirm the cross-reaction of commercial antibodies with the corresponding animal protein because commercial antibodies are frequently directed to human antigens or the antigens of a very narrow range of experimental animals. Due to this fact, and because Western blotting for testin had never been performed in dogs prior to the present research, three samples were preferred instead of one as generally needed. The Western blot results clearly showed bands with a mass corresponding to the testin protein, *i.e.* 47 kDa, in the three samples and thereby confirmed the correct performance of the test and the presence of this protein in dogs.

Analysing the results of our research, the most intense cytoplasmic reaction was observed in SCs and the cells of the reproductive epithelium, spermatocytes and spermatids in particular (Fig. 1A and B). This is in line with the results obtained in rats, where the authors observed the most intense reaction in these cells (5, 6, 12, 14), and it suggests that in dogs, as in rats, SCs could be responsible for the secretion of testin.

The different intensity of the protein expression in Sertoli cells of foetuses, puppies and adult (aged 2–6 years) dogs may indicate that the amount of testin increases with the development and maturity of these cells. The strongest expression of testin protein was observed in normal testes from mature dogs. At the same time, a reduced expression of testin was found in the testes of puppies, foetuses and in the atrophic areas of testes from aged dogs, suggesting that in the latter, SCs regress to immaturity. Similar changes in testin expression in testicular cells were observed in the rat. In very young, sexually immature rats, testin expression was weak or almost undetectable in SCs as well as in interstitial tissue (35). These findings are consistent with the results of the present study obtained from the puppies' testes. Moreover, in rats it was noted that, with age, the concentration of testin increases, being expressed by SCs, spermatocytes, and in sexually mature animals also in spermatids (35). These findings are consistent with those obtained in the present study and suggest that testin is conserved among species and that, as suggested in rats, it may also play a role in the spermatogenesis of canine species. A transient but drastic increase in testin accumulation was noted in rats between SCs and the head of elongated spermatids (12), as we also noted in the results of the present study. This may confirm the similar role of the protein in rat and canine testes more strongly. When the mRNA sequence in rats was elucidated, it was found that this protein is also present in prenatal organs (26). These results parallel those obtained in the testes of miscarried canine foetuses.

In aged dogs, where normal and atrophic tubules co-existed, a strong expression of testin similar to that detected in young adults was observed in SCs of normal areas. Conversely, in the Sertoli cells of atrophic areas, a weaker expression of testin was observed similar to that recorded in puppy testes. This was not reported in rats because atrophic testes were

not included in those studies. However, this finding, which we report for the first time, is interesting and consistent with previous studies which demonstrated that SCs in atrophic testes regress to an immature stage (10) and that SCs expressing markers of immaturity were in canine cryptorchid testes (23). These findings suggest that testin deserves to be further investigated in a larger number of canine atrophic testes, also including samples from cryptorchid dogs.

A weak expression of testin was also observed in interstitial endocrine Leydig cells, proving that the protein is not only present in Sertoli cells and in the reproductive epithelium. The results of the present study are consistent with those obtained in rats, where also a weak presence of testin was observed in interstitial endocrine Leydig cells (35). Although the role of testin in interstitial endocrine Leydig cells is still unknown, this additional proof that the testin protein is present in the dog not only in SCs but also in other cells opens the way for further research.

As mentioned in the introduction, the role of testin has constantly been investigated in recent years and its structure, as the gene coding for the protein, represents the focus of numerous studies, even if the function of this protein in humans is still poorly understood. However, in human medicine over the years, a role of testin in the process of carcinogenesis has also been noticed. The presence of testin has been detected, *inter alia*, in several carcinomas such as those originating from breast, endometrium, colon, lung, prostate, head and neck, and ovary tissue and also in squamous carcinomas (16, 25, 26). It has also been suggested that testin could play an important role in limiting tumour growth by arresting cell-cycle progression and invasion, inhibiting MMP2 secretion, this being inferred from the outcomes of *in vitro* and *in vivo* experiments (16, 25, 26).

This is the first study on testin in veterinary medicine, and canine testes were chosen because the dog is considered a better and more reliable animal model for the study of several human neoplastic and non-neoplastic processes (1, 3, 4, 11, 29). This is due to the longer life expectancy of dogs than rats and to the spontaneous occurrence of most of these pathological conditions in dogs, contrasting with the frequent necessity of experimental induction of these conditions in rats. However, leaving aside animal model appropriacy for human neoplasm research, the demonstration of the presence of testin in the testes of dogs of different ages and in foetuses will allow further studies not only on canine testicular pathology but also on the role of this protein in canine physiology, embryology and reproduction. Moreover, similarly to what has already been shown in human species, further studies on the expression of testin in the tumours of different organs could produce interesting comparative results.

The use of the Western blot method and protein homology detection allowed us to demonstrate the presence of testin in canine species and authenticate the immunohistochemical results, which were consistent with those reported in rats.

The results of the present study, obtained from testes of dogs at various life stages, encourage a further detailed analysis of the expression of testin in a larger number of canine testicular samples. Moreover, our results encourage investigation of the role of testin in canine biological and neoplastic processes. In addition, because the present results were obtained in a species sharing with humans a relatively long life expectancy, spontaneous neoplastic diseases, and exposure to one set of environmental risk factors, they could be the basis for future comparative studies.

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