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Contribution to and reconsideration of knowledge in small animal neonatology and gonadectomy in the dog

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Paper Collection

Physiology of the neonate and indicators for its well-being

Bodyweight at Birth and Growth Rate during the Neonatal Period in Three Canine Breeds

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Prostatic Neoplasia in the Intact and Castrated Dog: How Dangerous is Castration?

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Immunohistochemical investigation of the expression of estrogen and progesterone hormone receptors in extra-genital tissue in the female dog: preliminary results

Preface

In every field of research, advances in techniques and their availability permit us not only the discovery of new things but give us also the opportunity to review what has been believed to be true. Furthermore, there is a knowledge within specific groups of people based on experience rather than empiric results. Those two aspects of research and knowledge have given me the motivation and direction for the here presented PhD thesis. Within the field of neonatology in the canine species (a rather new field of research) consideration of the knowledge acquired by experience may be helpful. Breeders have shown to be versatile and adapting their habits depending on the experiences they had with their dogs in their kennel setting. Although this knowledge may not be confused with empirical data, it may help us researchers to understand what may be of interest and importance for both the animals and their breeders. Birth body weight and growth of the neonate is an easily measurable parameter which has been used by breeders to evaluate puppies' well-being for a long time. Studies then have shown that there is a correlation between birth weight and neonatal mortality. The approach used is a time-saving approach with a population composed of different breeds and conclusions drawn for all of them. Considering the variety of dog breeds a breed-specific evaluation of this parameter seemed necessary. Although such an evaluation is difficult and more time-consuming, the differences when comparing the results have shown the necessity of such a method.

The second and much bigger topic of interest, not only for my PhD thesis but for many researchers, practitioners and owners, is the impact of gonadectomy on the canine organism. This topic is complex, not only due to many unknown variables, but also due to its difficulty in attempting to investigate these variables. Elective gonadectomy has many proven positive effects on the animal and the relationship between owner and the animal. Studies which show a number of negative long-term health effects created major concern and doubt. It seemed necessary to me to investigate in depth, evaluate and compare studies on both noxious and beneficial effects, only to conclude that we know far too little to take a clear position for the whole canine population. Up until now no causal relationship with a pathway has been found between gonadectomy and long-term side effects such as orthopedic or neoplastic disorders. Due to this I attempted a first step on understanding the behavior of different extra-genital tissues towards sex steroid hormones by investigating the hormone receptor expression.

We may not presume that it will be easy to either confirm this relationship and its pathway, nor negating it, yet considering the impact of all the doubt and concern, it appears to be of utmost importance.

The people which contributed either directly or indirectly to my PhD thesis are scattered over different countries, yet their contribution was unimpressed by distance.
First of all, I would like to thank my family who has let me go, has believed in my decision to come to Italy also in moments when I didn't and, although far, has always found ways to be by my side. Further thanks go to Silvia and Nina for remaining present regardless the months that passed without seeing each other.
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Abstract

Part 1: Physiology of the neonate and indicators for its well-being

Veterinary neonatology is a rather young field of research. Important differences between the physiology of the neonate compared to the adult have to be taken into consideration in its clinical evaluation. The first topic of the present PhD thesis describes the current knowledge of neonatal physiology and different methods for evaluation of the newborns' viability. Methods such as the Apgar scoring system and blood gas analysis are successfully used in veterinary medicine. Birth weight and neonatal growth have been evaluated as parameters to estimate a puppy's risk for neonatal mortality. Although such a correlation has been proven, the populations used for this prove are composed of a high number of different breeds. To evaluate the usefulness of such an approach a comparison with a breed-specific evaluation has been made (Appendix 1). This comparison has shown important differences and the importance of breed-specific investigations of birth weight and growth of the neonate.

Part 2: Current knowledge on beneficial and noxious effects of gonadectomy

Elective gonadectomy is an important part of the daily veterinary practice. Although beneficial effects of gonadectomy on the dog have been proven, over the last two decades the number of studies reporting an increased risk for neoplastic and other disorders in the castrated animal increased. These reports, although predominantly retrospective, statistical investigations have caused concern and doubt within practitioners and owners. Easy access to information granted by the internet increased this doubt in the group of owners. Results of these investigations have been cited and described in scientific papers, as well as on non-scientific web-sites. In both, although most frequently in the latter, results of studies have been mentioned without describing or considering the study design, the population and the context in which the results have been presented. The lack of this information lead to premature conclusions which have an important impact on many stakeholders. It is therefore that the second part of the present PhD thesis presents a literature review of beneficial and noxious effects of gonadectomy. Prostatic neoplasia in the dog was used as an example of problematic differences in study design, population composition and interpretation of the results (Appendix 2). This part further presents the motivation for the experimental design of Part 3.

Part 3 and 4: Immunohistochemical evaluation of steroid hormone receptor expression in extra-genital tissue in the bitch motivated by reports on negative long-term health effects of gonadectomy

Hormone receptor expression has proven to be of importance not only in the research of a pathological pathway, but gave also indications on possible treatment options (e.g. human breast cancer). In veterinary medicine the research on hormone receptor expression is not yet as commonly used as in human medicine. The presence of hormone receptors in a tissue may be considered the basis for a direct effect of a hormone on a tissue. Nevertheless, their absence does not exclude the possibility that a certain hormone may not have an impact following a secondary pathway. Reports on effects of gonadectomy on extra-genital tissue gave reason to investigate the expression of Estrogen and Progesterone receptors. Immunohistochemistry is a viable method to evaluate hormone receptor expression. Samples of 14 bitches between the age of 2.1 to 16.7 years were taken within a maximum of 4 hours postmortem. Ten bitches resulted gonadectomized at the moment of death. Collected tissues include the right cardiac ventricle, the splenic hilum and the popliteal and retromandibular lymph nodes. Samples were formalin-fixed, paraffin-embedded and stained with hematoxylin and eosin for histological evaluation. Expression of progesterone receptor (PR) and estrogen receptor alpha (ERα) was evaluated with anti-human antibodies: monoclonal rabbit PR (Clone 1E2) and monoclonal mouse ERα (Clone EP1) using an automatic immunostainer (Ventana BenchMark GX, Roche Diagnostic). Reactivity with canine tissue was confirmed using dog uterus and ovaries showing strong positive nuclear staining for both ERα and PR. Tissues of 11 dogs were considered physiologic or mildly compromised, whereas 3 dogs presented neoplasia in heart (n=2) and spleen (n=1). IHC showed negative immunostaining within investigated tissues for neither ERα not PR.

Prior to drawing final conclusions, additional tests have to be performed to confirm the absence of ERα and PR expression. Despite the preliminary results in our study, we consider further research on hormone receptor expression necessary to understand the possible impact of gonadectomy on extra-genital tissue in the dog.

Contents

1. Physiology of the neonate and indicators for its well-being

1.1. Physiology of the neonate

The neonatal period is the time after birth during which important adaptions and development of vital functions take place. In veterinary medicine a clear definition on the length of the period is missing leaving some with the opinion that it describes the first 14 days of life[1], whereas others consider it the period until 4 weeks of age[2], and others again the first 21 days[3,4]. Especially in altricial species such as dogs and cats, the puppy finds itself after birth in the position to necessarily adapt to an unstable environment by developing the capacity to maintain a stable body core temperature[5], to start and maintain oxygen intake and gas exchange through the lungs[1], start alimentation through the gastrointestinal system, learn to defecate and urinate without external stimulus and much more[5]. It is due to the initial lack or decreased presence of these abilities that the canine neonate is highly susceptible to various problems throughout this period leaving it with a high risk of neonatal mortality.

The canine neonate has a low-pressure, low-volume, low-peripheral resistance circulatory system[6,7] which means, that to maintain sufficient peripheral perfusion the heart rate needs to be higher as well as the cardiac output[2]. Throughout the neonatal period bradycardia is not vagally mediated but rather a result of hypoxia and it is due to this that treatment with atropine has little to no effect[2]. The passage from oxygen supply through the placenta to intake and gas exchange over the lungs is surely one of the major adaptions a fetus has to face during birth and in the direct postnatal period. Low oxygen levels in the fetus are known to be one of the initiating factors for birth. This temporary hypoxia further induces the production of pulmonary surfactant which is essential for an adequate respiratory function after birth[8]. Reflex respiration may be induced in the first 3 days of life by stimulation of the genital or umbilical area by either the bitch or manually by a human; a factum that may come in handy during reanimation of weak puppies directly after birth[9]. The kidneys of a canine neonate are morphologically and functionally immature at the time of birth. Nephrogenesis and maturation of the kidneys continues for at least 2 weeks after birth[10]. This immaturity leads to a low urine specific gravity and the detection of protein, glucose and amino acids when a urine analysis is performed[11,12]. It takes between 6-8 weeks until urine concentration achieves values comparable with adult dogs and around 3 weeks to approach values of adult dogs in protein and glucose concentrations[11,13].

Changes within the gastro-intestinal tract (GI-tract) are marked as the puppy starts ingestion and digestion of milk. Most importantly the intestinal mucosa shows a time-dependent permeability[14]. Interestingly low body temperature has been linked to ileus development[15]. This

permeability, which decreases drastically after the first 12 hours of life, permits the passage of immunoglobulins provided by the colostrum[14]. A study published in 2012 concluded that the permeability for immunoglobins decreases as early as 4 hours after birth and is completely lost at 16-24 hours after birth. Furthermore, the results showed that immunoglobulin G serum concentrations were significantly higher in the group of puppies with ingested the colostrum within the first 4 hours after birth[16]. The timely ingestion of the colostrum and therefore of the immunoglobulins is considered vital for the puppy as it creates the first rudimentary immune system of the newborn.

Only 5-10% of the serum antibodies found in the canine neonate are provided through the placenta[17], compared to 25% in feline neonates[18]. Newborn puppies, both canine and feline, are therefor to be considered antibody deficient and immunologically incompetent[14,17]. These facts only underline the vital importance an adequate colostrum intake directly after birth to provide the newborns with passive immunity. Immunoglobin G, followed by immunoglobin A and M are the isotypes with the highest concentrations within the canine colostrum[2].

Newborn puppies are incapable of maintaining a constant body core temperature due to the lack of copying mechanisms such as shivering reflex and vasoconstriction and due to the combination of little subcutaneous fat and large body surface[17].

1.2. Neonatal mortality

Mortality of the canine neonate within the first 21 days of live ranges between 7% and 34%, with septicemia being one of the most frequent causes[19]. Reported mortality rates for canine puppies from birth until weaning age range between 20% to 40%, with the majority of deaths occurring within the neonatal period, that is within the first 21 days of life[20,21].

An investigation on neonatal mortality in the Boxer breed included 414 litters accounting for a total of 2629 puppies[20]. 571 deaths of puppies were recorded of which 147 puppies were stillborn and the majority (N=269 puppies) died within the first 21 days of life. Euthanasia due to the white coat color was performed in 102 cases and 53 puppies died between the 22nd and 50th day of life [20]. The mortality rate of Boxer puppies in this birth cohort study is reported to be 21.7%[20]. Although euthanasia in white boxer puppies is used, it may not be considered part of the incidence of neonatal mortality as it is a breed specific and breeder dependent decision rather than being a consequence of poor health of the neonate. Mortality rate was significantly higher in litters of less than 5 or more than 8 puppies compared to litters with 5 to 8 puppies[20]. Causes of death were divided in congenital abnormalities (cheilopalatoschisis, atresia ani and others), inflammatory disorders (sepsis, pneumonia, other infected organs or combination of

infected organs) and non-inflammatory disorders (asphyxia, malnutrition and others). Of all recorded deaths, excluding the ones euthanized due to white color, 77 died due to congenital abnormalities, 102 due to inflammatory disorders, 66 due to non-inflammatory disorders and 224 due to unknown reasons [20].

Within a very large studied population of over 200,000 puppies of 248 breeds followed until being sold, the mortality rate of puppies was 13.4% with stillbirth accounting for more than half of all deaths. This means, that of all litters born, 31.5% had at least one stillborn puppy or at least one neonatal/pediatric death[22].

Another study showed by describing neonatal mortality for specific day ranges rather than for the whole first 3 weeks in 744 puppies of large breeds[3]. Stillbirth was found most frequently (n=81) followed by decreasing mortality from the period of 0-3 days (n=29) of life until the age of 15-21 days (n=2). Big litter sizes and low birth weight of the puppies have been associated with increased risk for mortality, with low birth weight being the most frequent cause of death within the first 3 days of life[3].

Due to the high rate of neonatal mortality in dogs, indicators and risk factors for neonatal mortality are of great interest. Low APGAR scores, low blood glucose levels and low birth weight have been linked to an increased risk for neonatal mortality[23]. Other reported significantly influencing factors on neonatal mortality are increasing age of the mother, parity of the mother (with the first litter being at highest risk) and breed[4]. Furthermore, puppies born in a litter with at least one stillborn puppy are reportedly at a higher risk for neonatal mortality[4].

1.3. Evaluation of the neonate and early indicators for neonatal distress

1.3.1. APGAR

The APGAR scoring system, developed in 1952 to evaluate newborn babies, encompasses 5 parameters: Appearance, Pulse, Grimace, Activity and Respiration[24]. Each parameter is given points on a scale from 0 to 2. If the sum of these points is less than 3, human newborns are considered critical[25] and require medical attention. The APGAR scores main function is prediction of mortality rather than being an indicator for long-term damages[26,27] and has been considered to be a more viable predictor for survival than umbilical artery blood gas measurements[28]. The viability of a modified version of the APGAR-scoring system has been evaluated in a population of 193 puppies of 42 litters[29]. Parameters evaluated included heart rate, respiratory effort, reflex irritability, motility and mucus color. Furthermore, body temperature was recorded and behavioral patterns (searching for the mammary gland, suckling

and swallowing reflexes) have been classified as either present or absent. 27 puppies were stillborn and only 9 of 166 puppies which were born alive died within the first 24 hours of life. Puppies which did not survive until 2 hours after birth had an APGAR score of 0 to 3 or 4 to 6. Compared to the group of puppies with an APGAR score of 7 to 10, the difference was statistically significant[29]. Although type of delivery within one study had no impact whatsoever on the mortality rate of the puppies[29], another investigation reports a lower vitality with a lower APGAR score in puppies born by c-section compared to puppies born vaginally[30]. This difference changed after the first 5 minutes of life with puppies born by c-section coming to develop satisfactory scores in the reevaluation[30]. In 2015 neonatal reflexes were evaluated in one study by a scoring system as either weak (0 score), moderate (1 score) or normal (2 score)[31]. Although the evaluation of suckling has been recorded as present or absent already in the study of Veronesi et al[29], Vassalo and coauthors[31] further included rooting and righting reflexes. A proven direct and statistically significant relationship between APGAR score and neonatal viability[32] and the possibility to perform this non-invasive method with some training but without specific material makes it a valuable tool for early evaluation of puppies in the need of medical attention.

1.3.2. Blood gas analysis

Measurements of venous blood gas parameters (e.g. pO_2 and SO_2, pCO_2) have been reported in the evaluation of the canine newborn. Blood was drawn directly after birth from the jugular vein[30,31]. Blood lactate levels have been reported to be a useful tool in obtaining information on the clinical condition of a canine newborn[33]. Although measurements of certain parameters may give indications on the well-being of the puppy, there are some aspects that need to be considered: a) results are dependent on the timing of sampling, b) results may differ between puppies born by eutocic parturition or c-section[31], c) although few blood (0.1ml[31]) is needed, the sampling process in itself is nevertheless to be considered invasive. Sampling blood from a newborn puppy is not easy due to the size of the veins. Considering the incompetent immune system special care should be taken on disinfection when sampling. Although usefu,l not every practitioner has the dexterity and possibility to perform a blood gas analysis in the immediate vicinity to parturition. Considering that the APGAR scoring system is non-invasive, easily understandable and does not need a laboratory it seems the preferable evaluation tool.

1.3.3. Birth weight and growth during the neonatal period

Measurement of the canine newborns birth weight is easy and recommended in every case of birth in pet dogs. At the moment a total of 349 canine breeds are recognized by the Fédération Cynologique Internationale (FCI) which makes the dog a species with a wide variability of phenotypical appearance, including body weight. Low birth weight and low weight gain during the first days of life have been linked to neonatal mortality[3,23,34–36] and may be considered either a risk factor or an early indicator for health problems in the puppy. Studies which show a correlation between low birth weight or low weight gain and neonatal mortality evaluated a population composed of different breeds grouped according to the expected adult body weight of the breed. Until today, no official, generally accepted rule is available on how to create breed and weight groups, which leads to important differences between studies. The Tibetan Terrier for example has been included in two studies, once as a dog of small breed[23] and once as a dog of medium size[36]. The decision to investigate puppies birth weight and growth during the first 3 weeks of life in a breed specific manner was motivated by both the reported importance of birth weight and weight gain for the identification for puppies at risk as well as due to the above-mentioned differences in the grouping of breeds according to their body weight. Weighing of puppies is probably the easiest tool applicable by and useful for the breeder without veterinary assistance. Yet prior to the identification of puppies at risk, physiological birth weights and physiological daily gain of the breed must be known. I used therefore a population of puppies of 3 breeds (Lhasa Apso, Tibetan Terrier and Bernese Mountain Dogs) to evaluate physiological birth weight and influencing factors and included puppies of 2 breeds (Tibetan Terrier and Bernese Mountain Dog) in the evaluation of physiological neonatal growth (Appendix 1). All evaluations have been performed in a breed specific manner and comparisons between breeds have been made. Comparing my results with previously published results the importance of breed specific evaluations of the parameter became evident.

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2. Current knowledge on beneficial and noxious effects of gonadectomy

2.1. History and evolution of society towards gonadectomy

Gonadectomy in the dog is a routinely performed surgery with the goal to remove the gonads of either male or female dogs in order to obtain infertile animals with reduced to no sexually motivated behavior. Since the beginning of its routine application, gonadectomy and perception of it underwent revolutionary changes. In the beginning dogs, especially females were gonadectomized indiscriminately of their size, age, behavior or health status. This may be attributed to the intense search of a method to control unwanted offspring as well as the lack of contradicting information concerning both surgery and the long-term effect on the health of the subject. With increasing amount of research in the area of veterinary medicine studies showed beneficial effects of castration on behavior and health of the animal. Apart of the control of unwanted reproduction, protective effects have been proven towards sex-specific pathologies such as endometritis, pyometra and benign prostatic hyperplasia[1] (Root Kustritz, 2012). Furthermore, studies showed an increase in lifespan of gonadectomized dogs towards their sexually intact peers[2,3]. During the 1990 and early 2000 studies further provided information on a protective effect of early castration (prior to the first heat) on the development of mammary carcinoma[4]. These findings initially appeared very promising and the practice of prepubertal castration was advertised and performed more frequently. Only in 2012 this practice was revised when a systematic review of the data used to arrive at the above-mentioned conclusion, showed, that the prove was rather weak[5]. Furthermore, studies showed that the removal of the gonads prior to the first heat and therefore prior to maturity may have side-effects on the whole organism, such as baby-fur, increased growth of the long bones[91] and following this a presumed increased risk of orthopedic disorders. After the publication of this systematic review attitude towards prepubertal gonadectomy changed and the majority of veterinarians, both practitioners and researchers, advised for the surgery to be performed between first and second heat which ensures the maturity of the bitches' body. Already in the late 1980s studies have been published on other side effects of gonadectomy on the canine organism for both the male and the female, yet in the beginning these findings got few attention. Over the years the number of reports increased, linking neoplastic[6–12] and orthopedic disorders[13], urinary incontinence[14], obesity[15] and behavioral changes[12,16,17] to surgical castration. The increase in ease of access to information for both professionals and layperson may have contributed to the momentary situation in which routine gonadectomy in the dog causes doubt and concern. More than 80%

of 630 interviewed owners in the UK have been reported to nevertheless agree with elective gonadectomy[18].

2.2. Techniques of surgical castration and alternative options

With advances in surgical techniques regarding gonadectomy also research on possible alternatives increased. Castration is by definition the surgical removal of the gonads; precisely both testicles in the male or both ovaries in the female[19]. Chemical castration or functional castration, although often used may therefore be considered a confounding term as it is used to describe pharmacologically induced infertility without removal of the gonads[20].

2.2.1. Pharmacological methods

GnRH agonist slow-release implants are commercially available as a pharmacological method to achieve infertility in the male dog. Implants are positioned after thorough clinical exam in the subcutis of the male dog. After application an initial "flare up" results in an initial increase of testosterone secretion[21]. Once the "flare up" passes testosterone concentrations decrease and azoospermia is achieved[22]. The duration of the implant is dependent on various parameters but is reported to be at a minimum of 180 and 400 days for a 4.7mg and 9.4 mg Deslorelin implant, respectively[23]. The effect has been reported to be reversible[22].

2.2.2. Other invasive methods

Vasectomy is a commonly performed routine surgery in human medicine and is considered less invasive than a tubal ligation. In veterinary medicine vasectomy in the dog has been described, yet is not as commonly performed as orchiectomy. Surgical vasectomy consists of the bilateral occlusion and/or partial resection of the deferent ducts[24]. Although this procedure makes the male incapable of reproducing, the continuing production of testosterone leaves sex characteristics and behavior unchanged as well as the risk for androgen-dependent diseases[24]. As a further evolution of the technique, keyhole (laparoscopic vasectomy)[25] and pinhole (percutaneous spermatic cord ligation)[26] approaches have been described. Intra-testicular injections of zinc gluconate[27] and calcium chloride dihydrate ($CaCl_2$) solely or in combination with dimethyl sulphoxide[28]or with ethanol[29] have been described for achievement of sterility in male dogs. Injection of $CaCl_2$ resulted in temporary sterility. Both reduction of spermatozoa and side effects are reported to be dose-dependent[30]. Although the above-mentioned techniques result in sterility the effect was not permanent. Furthermore, alternatives to assure infertility,

also only temporary, in the bitch are under investigation but not yet widely available. Gonadectomy remains the only permanent solution to avoid unwanted reproduction in both female and male dogs.

2.2.3. Gonadectomy

As well as in the theoretical aspect also the practical aspect of gonadectomy saw an evolution over the last two decades. Castration of the male, due to its simplicity of access remained the same. The surgical approach of castration of the female instead changed in an important matter. Initially the castration of the bitch included the removal of the gonads (ovaries) as well as the removal of the uterus until dorsally of the cervix. This approach resulted in a relatively long incision. Over the years studies have shown, that the removal of the ovaries, if the gonads are removed in toto, is enough to provide infertility, a protective effect towards endometritis, pyometra and other uterine pathologies and a possible protective effect towards the development of mammary carcinoma[31]. Considering that at this time urinary incontinence after castration was treated with estrogens, professionals remained skeptical towards ovariectomy as they suspected a possible induction of uterine pathologies by treatment of urinary incontinence[32,33]. As with time different drugs and treatment options[34] for urinary incontinence came on the market and studies showed that also with the use of estrogens, the risk of inducing uterine pathologies is low[35], the attitude changed and ovariectomy became recognized as the better option to castrated females. Removal of the uterus became an extension of surgery in case of presence of uterine pathologies at the time of surgery such as glandular cystic hyperplasia of the endometrium. The change in surgical approach resulted in a smaller incision, a shorter surgery and therefore a shorter anesthesia. With the increased application of laparoscopy gonadectomy was used as an entry level surgery for veterinary surgeons starting their education in laparoscopy[25]. The anatomical location and the fact that there are only two major vessels to ligate made it the perfect intraabdominal surgery to get accustomed to the handling and use of laparoscopic techniques and instruments. Today laparoscopic ovariectomy evolved to a single port surgery through which in case of necessity also the uterus may be removed[36,37]. Furthermore, reports were published on the possibility of a laparoscopic ovariectomy through the vaginal canal[38].

2.3. Reported long-term side effects of gonadectomy in the dog:

2.3.1. Urinary incontinence

Urinary incontinence per definition is connected to a sphincter deficiency which results in the unconscious and unwanted loss of urine[39]. This deficiency may have many different causes and prior to diagnosing a castration-related incontinence other possible causes have to be excluded. Common causes for urinary incontinence are bacterial infections of the lower urinary tract, malformations and other disorders[40]. Cases of lower urinary tract infections present often with other symptoms such as dysuria, hematuria and pain during urination. Diagnosis or exclusion of such a disorder from the list of differential diagnosis may be performed by a thorough collection of the clinical history and a urine sample and analysis. Once all possible causes are excluded from the list of differential diagnosis, castration-related incontinence may be considered as final diagnosis. Incontinence usually manifests around 2-5 years after surgical castration and is more frequent in females than it is in males[40–42]. Age at castration had no impact on the development of incontinence[41]. Studies excluded a correlation between surgical technique and the development of incontinence[43] yet obesity at the time of surgery is considered a predisposing factor[41,44–46]. Furthermore, a breed related increased risk was proposed[43]. Early treatments of urinary incontinence included the use of estrogens[47]. The use of such drugs in ovariectomized bitches should be evaluated carefully as side effects on a uterine level may occur [32,33], yet other studies negated such a risk[48]. Nowadays different methods of treatment are available and are usually applicated in a cascade. Firstly, pharmacological treatment with drugs on the basis of phenylpropanolamine are used[49]. Results are promising, yet patience is required to find the minimal active dose for each individual. In case pharmacological treatment is without success, surgical techniques have been proposed with the scope of increasing the competence of the sphincter[50–54]. Although some studies clearly state a correlation between the development of incontinence[40,44] and gonadectomy a systematic review of 2012 concluded that the evidence was weak[55]. Nevertheless, studies have shown the presence of estrogen receptors in the proximal urethra[56,57] and sustain due to this finding a causal relationship between the lack of estrogen due to gonadectomy and the incompetence of the sphincter muscle[40,58].

2.3.2. Obesity

Obesity in the dog is a common disorder[46]. A dog's body condition (cachectic to severely obese) may be described using a body condition score (BCS) which ranges either from 1-5[59] or 1-9[60,61]. Although a high body condition score at the time of castration has been linked to an increased risk of the development of urinary incontinence[41,44,45], changes in the body condition have been

proposed as a long-term side effect of castration on its own[15,62]. The lack of sexual hormones and therefore, the lack of sexually motivated behavior was considered to be the cause of a diminished energy requirement of the feline organism[63–66], a similar process may be hypothesized also in the dog. Other studies on the other hand report a change in feeding behavior leading to the combination of increased food intake and decreased activity[67]. Although this may easily be possible, obesity in the dog is highly dependent on the owner and the dogs' environment, contrary to the other possible side effects of castration which are independent from such factors[68]. Bitches and neutered males have been reported to be at higher risk of developing obesity[69]. In any case the owner should be informed prior to the surgery that a tendency towards obesity may be present in the castrated dog and adequate measures may be necessary. Especially as obesity has been linked to increased risk of the development of other disorders such as orthopedic problems[62]. Producers of commercially available dog food took this possible correlation into consideration and provide the possibility of less-energetic dogfood used for maintenance, usually promoted under the name "sterilized". Although experiences with such products have been positive it is nevertheless advisable for the practitioner to inform the owner and explain his/her responsibility towards the development of obesity. Regardless of an increased risk, castration should not become an excuse for the development of obesity in pet dogs.

2.3.3. Neoplastic disorders

2.3.3.1. *Osteosarcoma*

Although the reported incidence of osteosarcoma is as low as 1.24%[70], the neoplasia is reported to have a high malignancy rate and is the most common tumor type affecting the skeleton[71]. The Rottweiler and the Great Dane are breeds at particularly high risk for osteosarcoma development[70]. Furthermore, a strong genetic component for the risk of development has been reported[72]. Reports on the influence of age are contradictory with studies reporting either only moderately influenced by age[70] or showed an increase in diagnosis until the age of 10 years[8]. Also regarding sex, reports are incongruently describing either no sex related[8] differences or an increased risk for female versus male dogs[6]. Agreement between studies may be found instead on the impact of gonadectomy on the risk for osteosarcoma[6,8,70]. Castrated individuals (both female and male) have been reported to be at a higher risk for osteosarcoma development compared to their intact peers[6,8,70]. On evaluation of the lifetime gonadal exposure of dogs, a study in 2002 concluded that dogs castrated early in life are at the highest risk and that for each month a dog remains sexually intact the osteosarcoma risk reduced for 1.4%[6]. Nevertheless,

readers have to keep in mind, that this study was conducted including exclusively Rottweiler which are known to be a breed of increased risk. One study described castrated males as being at a higher risk compared to intact males for osteosarcoma development[70]. Early neutering together with high height and high weight have been reported to be one of the risk factors for osteosarcoma[73,74].

2.3.3.2. Hemangiosarcoma

Hemangiosarcoma (HAS) has been described to occur with an incidence of 2.8% most frequently in soft tissues and the skin and only in 5.93% of cases in the blood or hematopoietic system. Females compared to males presented with a lower risk for HAS development, yet on evaluation of the neuter status, gonadectomized females were at a higher risk[70]. In 1999 the incidence of cardiac tumors was reported as 0.19% with HAS being the most commonly diagnosed cardiac neoplasia[10]. Within this study castrated males and females have been reportedly at an increased risk for HAS development than their intact peers. No information is given on the time of gonadectomy in respect of the time of diagnosis of the tumor [10]. In contrast to previously reported low incidence of HAS, in 2018 a study including only Golden Retriever dogs reported that 22.64% of all cancer related deaths were caused by HAS[7]. The study in question gives no information on the percentage of castrated individuals in the HAS affected group yet reports that in the group on Golden Retriever not dying of cancer, intact females lived significantly less long than castrated females[7]. As has been mentioned for osteosarcoma early spayed animals have been considered as the group at highest risk for different suspected side-effects of gonadectomy, yet in 2013 a study conducted on a population of Golden Retrievers showed a significantly higher risk for HAS for late-neutered females compared to intact and early-neutered bitches[13]. Such differences were not observed in the group of male individuals[13]. Such a distinction between early and late-neutered individuals has not been observed within a population of Viszlas, yet also in this study castrated individuals have been mentioned to be at increased risk for development of HAS[12]. Although studies on HAS gave more information on the sexual status of their population than many other studies by dividing the group in early-neutered, late-neutered and intact animals, differences are found on when an animal is considered neutered early or late. Furthermore, the above-described studies include mostly populations of only one breed and do not provide information on the castration-diagnosis-interval (CDI).

2.3.3.3. Lymphoma

The incidence of lymphoma in the dog ranges between 1.2%[9] and 4.35%[70].

As is observed in human medicine, also within a canine population, females have a lower incidence of lymphoma than males, as long as no significant hormonal changes occur (either by menopause in the human or gonadectomy in the dog)[9]. A study reports an increased percentage of diagnosis in castrated females compared to intact females, yet a decreased percentage of diagnosis in castrated males compared to intact males[9]. Overall lymphoma has been diagnosed most frequently in castrated females[9]. On the contrary others reported an increased risk for both castrated females and males compared to their intact counterparts[70]. In a population of Golden Retrievers lymphoma has been the second most common cause for cancer related deaths accounting for 18.4%[7]. Also, within this population lymphoma was more frequently diagnosed in castrated males and females[7]. Comparison of a population of Golden Retrievers and Labrador Retrievers instead illustrated a very interesting difference. Although the finding of castrated females and males (in particular animals castrated between 6-11 months of age) being at a higher risk in Golden Retrievers is consistent with other reports, such an impact could not be observed in Labrador Retrievers[75]. In 2013 male and female Golden retrievers neutered early (<12 months of age) in life were reported to be of higher risk for lymphosarcoma diagnosis[13]. In Viszlas instead males and females neutered at >12 months of age were the most frequently diagnosed cases of lymphoma[12]. When comparing these studies important differences become obvious, especially concerning the breed specific evaluations. Although the study by Villamil et al[9] included individuals of different breeds, the other above-mentioned studies concentrated on populations composed of either one or maximum two breeds. Within these comparisons, disagreement between the effect of early and late gonadectomy become evident as well as disagreements on the effect of gonadectomy in general. Although providing more or less detailed information on the time of gonadectomy, all studies lack again a castration-diagnosis- interval.

2.3.3.4. Mast cell tumor

Mast cell tumors (MCT) are cutaneous neoplasia with a reported incidence of 6.5%[70] and present the most frequently diagnosed cutaneous neoplasia in the dog[76–78].

Both male and females which have been castrated are reported to be at increased risk for MCT diagnosis[70]. Female Golden Retrievers have been reported to be of increased risk for MCT regardless the age at gonadectomy; an observation that could not be found in Labrador Retrievers[75]. Similar results were found in a different population of Golden Retriever in which

late castrated females (>12 months of age) were found to be more frequently diagnosed than early-castrated or intact females; no such difference has been observed in the group of males[13]. Regarding a population of Viszlas, females and males were equally likely to be diagnosed with MCT, yet castrated individuals had a significantly higher risk to be diagnosed with MCT than their intact counterparts[12]. Within a population composed by 114 pure breed castrated females were significantly more likely to be diagnosed with MCT than their intact peers, yet the increased risk for castrated males did not significantly differ from their intact counterparts[11]. Both Labrador and Golden Retriever are considered breeds of increased risk for MCT[11], a finding that makes the differences in results concerning the impact of gonadectomy within these breeds even more interesting. As has been underlined for other neoplastic disorders, also in the case of MCT reports of castration-diagnosis-interval are missing and findings are somewhat contradictory.

2.3.3.5. Prostatic neoplasia

The canine prostatic neoplasia (CPN) is the only neoplasia developing within the genital apparatus in the list of neoplastic disorders which are presumed to be influenced by gonadectomy in the dog. Furthermore, it is one of the rarest within the list of the described tumors with a reported incidence of 0.35%[79]. As for the above-described neoplastic disorders, an increased risk for castrated animals has been reported also for CPN. Authors went even further and attributed castration the capacity to favor tumor progression[80]. In the human male, prostatic cancer was the cause for >400,000 deaths in the United States of America in the year 1995 and was thereby considered the most frequently diagnosed non-cutaneous tumor[81]. Canine prostatic neoplasia and its human counterpart share many similarities which made the dog a species considered to be a valuable model[82]. Not only due to this, literature on prostatic cancer in the dog, regardless its low incidence grew fast. Reports on possible risk factors increased and very early compared to other neoplastic disorders, the possible impact of gonadectomy on the neoplasia development was mentioned and investigated. The high number of studies investigating this possible impact as well as the difficulty in hypothesizing a possible causal relationship between the decrease of testosterone and the development of a neoplasia in a generally considered androgen-dependent organ was the reason to evaluate the various studies, compare them and provide thereby an in-depth review on the current knowledge on CPN in general and the possible effect of castration in particular. The published review may be found in Appendix 2.

2.3.4. Orthopedic disorder

Hip dysplasia (HD) and tear or rupture of the cranial crucial ligament (CCT) are orthopedic disorders with a reported incidence of 1.8% and 1.7%, respectively[83]. Although these disorders are not life-threatening, their presence may have an important impact on the quality of life due to pain[84]. Furthermore, treatment of both conditions may be cost intensive for the owner and especially in dogs of large breeds either condition may be a cause for the owner to opt for euthanasia[84]. Whereas the development of hip dysplasia has an important genetic component, CCT is found mainly in younger dogs following a traumatic event or due to degenerative processe[85]. Large breed dogs are more likely to be affected by CCT than dogs of medium or small breeds[86–88]. Gonadectomy had been reported to be a risk factor for the development of both hip dysplasia and tear of the cranial crucial ligament[13,75,86–90]. A study in 2019 evaluated different motives for dismissal of service dog candidates and its possible correlation with the sexual status of the individuals. Most dogs in this population were males. A total of 25 dogs was dismissed for orthopedic disorders. The majority (n=15) of these dogs were neutered before 7 months of age. The most frequently found breed in this group was the Labrador Retriever. Reasons for dismissal due to orthopedic disorders include hip and elbow dysplasia, osteochondrosis dissecans, crucial ligament disease and other degenerative joint diseases[90].

To evaluate the impact of prepubertal castration on the skeletal development of puppies, ovariohysterectomy was performed in 11 female puppies of 10 weeks of age and 10 puppies were castrated incompletely (one ovary remained intact). Monthly measurements until the age of 24 weeks showed an increased ulnar and radial length compared to incompletely castrated and intact animals, yet the difference was not statistically significant[91]. Furthermore, puppies in the ovariohysterectomized group were heavier yet also this difference was not statistically significant[91].

Occurrence of hip dysplasia and curcial ligament rupture in Golden Retrievers and Labrador Retrievers and its possible correlation with the individuals sex status have been evaluated in two studies[13,75]. Findings in the group of Golden Retrievers showed an increased occurrence of hip dysplasia in early- neutered (<12 months of age) males compared to late neutered (>12 months of age) males and their intact counterparts; the BCS did not differ. These findings could not be observed in females[13]. The same increased occurrence of hip dysplasia in males neutered <6 months and between 6 and 11 months of age could be observed in a different population of Golden Retrievers. The BCS of neutered males with HD was higher (6.0) than in the group of intact males with and without HD (median 5.0). On the contrary to the above-mentioned population, in the present one also females showed to have a higher occurrence of HD when

neutered <6 months of age, yet the difference to their intact peers was not statistically significant[75]. Within the group of Labrador Retrievers no differences were observed in males, yet early neutered females were affected more frequently by HD. Interestingly, neutered bitches with HD showed a BCS of 5.5, whereas their intact peers with HD had a BCS of 7[75]. Cranial crucial ligament tear (CCT) is a serious but common injuria in dogs. Regarding the Golden Retriever population of 2013, early-neutered dogs were significantly more likely to be affected by CCT than their late neutered and intact peers in both females and males. No significant differences could be observed in BCS between neutered animals with and without CCT[13]. Coming back to the Golden Retriever population investigated in 2014, CCT was found significantly more frequently in males neutered at <6 months and between 6-11 months of age and in neutered females regardless of the time of castration[75]. The BCS of castrated males and females with CCT was higher than the one of their respective castrated an intact peers without CCT. In Labrador Retriever CCT was found significantly more frequently in males castrated <6 months of age; the BCS of castrated males and intact males with CCT did not differ[75]. In females CCT was found more frequently in animals castrated <6 months of age but on the contrary to males, the difference was not statistically significant[75]. Gonadectomy at an early age was linked not only with an increased occurrence of hip dysplasia but also with a diagnosis at a younger age. Nevertheless, owners of these dogs did opt less frequently for euthanasia which is hypothesized to be due to a less severe form of hip dysplasia in these subjects[92].

2.4. Studies and their limitations

The increased risk for tumor development in castrated animals includes prostatic carcinoma[80], lymphoma[93], hemangiosarcoma[10], mastocytoma[94] and osteosarcoma[6,8]. With exception of prostatic carcinoma, the reported neoplastic disorders develop in extra-genital tissue. This fact makes it difficult to hypothesize and then prove a causal relationship between castration and its increased risk for development. Studies on the subject may differ in study design (retrospective analysis of data[95] versus histopathological evaluation[96]) and in inclusion criteria of the population (e.g. number of breeds[7,12,13,75]) yet most of them have one thing in common which may be considered the most crucial limitation. Most studies include in their investigation animals which are castrated and animals which are sexually intact at the time of diagnosis and/or death. Animals which resulted without gonads at the moment of diagnosis were indiscriminately inserted in the group of "castrates" without consideration on the castration-diagnosis-interval (CDI). It is this interval or better the minimal necessary interval between castration and diagnosis which may allow researchers and practitioners to evaluate the risk and its possible increase realistically. As most neoplastic disorders are not acute but are composed of different changes on a cellular level over a certain period of time, it seems incorrect to conclude, that, in animals which have been castrated only a couple of months or less prior to diagnosis, castration was the initiating factor of carcinogenesis. Although this may seem obvious and logical, only a handful of studies describe both the CDI and the minimal CDI for inclusion in the group of castrated animals for their population[80,95].

Furthermore, studies which have been conducted on the basis of data collected from one specific breed, are frequently used to provide information without stating clearly that the conclusion was drawn from results obtained from a single breed population. This observation brings us to the second important limitation, which is the lack of context in published scientific reviews as well as in non-scientific articles. Due to possible editorial limitations of the length of a scientific review, the author of a review on possible side-effects of gonadectomy is bound to keep each paragraph short, meaning that the information which may be provided on a single side-effect is limited. This limitation results in reports of increased risks without the necessary information on the study design, the studied population and inclusion criteria. This lack of context is one of the main reasons for premature conclusions.

Especially on prostatic carcinoma in the dog, the number of studies is relatively high and the differences between these studies are immense. Although the incidence of prostatic carcinoma in the dog is low, it was this discrepancy that made me chose this neoplastic disorder and

possible side-effect of castration as an example to show what we know, what we think to know and how big the difference between the one and the other is.

2.5. Reported positive effects of gonadectomy in the dog

2.5.1. Pyometra

Pyometra describes a pathologic accumulation of purulent material inside the uterine lumen. This production and accumulation of pus usually follows a bacterial infection[97]. For a long time pyometra was seen as a component of a complex pathology often referred to as cystic endometrial hyperplasia (CEH)-pyometra complex. Only after years of research, veterinarians agreed, that pyometra may be present within a subject contemporaneously with CEH, yet the presence of CEH is not a necessary component in the pathoetiology, of pyometra[98]. The pathology is mostly seen in bitches of more than 8 years of age[99] and is a pathology typically found during the diestrous[100]. The disorder may lead in rare cases to death in endotoxic shock[101]. In general, two types of pyometra may be distinguished dependent on the if the cervix is open or closed at the time of pus accumulation. Whereas a pyometra with open cervix may be easily suspected due to the purulent vaginal discharge[102], a closed pyometra presents with generalized but unspecific symptoms such as anorexia, depression, fever, polydipsia/polyuria and abdominal pain[102]. It is therefore that pyometra should be included in the list of differential diagnosis in every case of above-mentioned unspecific symptoms in intact, postestrous bitches[103]. Diagnosis is made by ultrasound examination of the genital tract[103]. Especially in the cases of closed cervix pyometra the thickness of the uterine wall should be evaluated due to the risk of rupture in extreme cases of accumulation. Furthermore, the ultrasound may reveal cystic formation of the endometrium[104]. Ovariohysterectomy remains so far, the most effective treatment of pyometra[105], yet animals in poor general conditions should be stabilized prior to attempt surgery[103]. In cases of pyometra in young bitches which are destined for reproduction treatment with Aglepristone is possible[106] with or without prostaglandin F2α (PGF2α)[107]. Bitches should be mated in the following estrous cycle to avoid recurrence of the pathology[103]. The protective effect of bilateral ovariectomy on the development of the disorder has been described already in the 1930's[108] and is especially common in countries in which elective gonadectomy in the bitch is not routinely performed[105].

2.5.2. Neoplasia of the female genital tract

Neoplastic disorders in the genital tract of the bitch are rare conditions. Possible sites of neoplasia include the ovaries, the uterus and the vagina with ovarian tumors being the most commonly found. Reported incidences of ovarian neoplasia range from 0.5% to 6.2%[109–113]. Ovarian tumors have been reported to cover 0.5% to 1.2% of all diagnosed canine neoplasia[114]. Ovarian tumors may be subdivided into epithelial, sex cord-stromal or germ cell tumors. The

most commonly found epithelial tumors are adenomas or adenocarcinomas. The most common sex cord-stromal tumors are granulosa cell tumors and dysgerminomas are the most common germ cell tumors. Overall the most commonly diagnosed tumors are granulosa cell tumors[71]. The risk for ovarian tumors increases with age as has been reported for many different neoplastic disorders in the dog[115]. Breed predispositions have been suggested[109,116,117]. Diagnosis of ovarian neoplasia is difficult as symptoms may be absent for a long time. Symptoms of big ovarian tumors include ascites[114] and in case of steroid hormone producing masses such as granulosa cell tumors irregularities in the estrous cycle may be noted[118]. Uterine neoplasia is a rather rare condition in the bitch with leiomyomas being the most commonly diagnosed type of neoplasia[119]. Leiomyomas are mesenchymal benign neoplasia affecting the smooth muscles[120]. Only in rare cases the muscle layer may be affected by leiomyosarcomas a malignant mesenchymal neoplasia. Both types of tumors rarely create symptoms until the tumor is of important size. In case of leiomyomas the removal of the tumor and the uterus in toto is considered a curative surgical treatment.

An incidence of 0.85% has been reported for vaginal neoplasia[120]. An association between the development of vaginal tumors and ovarian follicular cysts, mammarian pathologies, estrogen secreting neoplasia and cystic endometrial hyperplasia has been suggested[121]. Considering that leiomyomas are the most commonly diagnosed vaginal tumors the prognosis after surgical excision has been reported to be good[120]. Cases of vaginal neoplasia in gonadectomized bitches are extremely rare[120,121]. Clinical signs may include vaginal discharge (purulent or hematic), swelling of the vulva or perineum and in rarer cases urinary or fecal tenesmus[120].

2.5.3. Mammary carcinoma

The mammary gland is one of the three most common site of neoplasia diagnosis in the dog[71,123,124]. In Italy mammary tumors have even been described to account for 70% of all cancer cases[125]. As for most neoplastic disorders also, mammary neoplasia is found more frequently with increasing age[125]. As early as in 1969 a study stated that bitches which were castrated prior to the first heat were considered to have 0.5% of the mammary tumor risk, bitches which were castrated between the first and second heat had 8% and bitches which were castrated after the second heat or later had a risk of 26%[4]. Reports like this increased the application of prepubertal castration. In 2012 a systemic review of concluded that the initial presumption that early castration may decrease the risk of mammary tumors notably is poorly proven[5]. Nevertheless, neutered bitches have been reported to have a lower risk for development of mammary neoplasia than intact bitches[2,70]. Studies on mammary tumors after

2012 further sustain that the development of mammary tumors is an event programmed early in life and early castration remains the only effective tool to prevent the occurrence of these tumors[126]. Regarding body size, bitches of small size have been reported to be more frequently affected by mammary tumors[70]. English Springer Spaniels, Dobermanns and boxers have been reported to have an increased risk for mammary tumor development[127]. Mammary tumors remain without clinical signs for a long time. Owners or practitioners may nevertheless diagnose its presence early by regular palpation of the mammary gland[126]. Tumor size, spread to regional lymph nodes and distant metastasis are of prognostic value[126]. Until now numerous different histological types are known for both malignant and benign tumors[128]. Ductal carcinomas, the most commonly diagnosed mammary carcinoma, may further be divided depending on their degree of malignancy[126]. Fine needle aspiration biopsies may be used for diagnosis yet excisional biopsy is to be preferred[129]. Surgical removal is considered the first choice of treatment for mammary tumors with the exception of inflammatory carcinomas[128].
At the momentary stage of research, the extent of surgical intervention depends on numerous factors, such as the dimension of the tumor, the number of affected mammary complexes, and the location of the tumor[126]. Chemotherapy may be the treatment of choice in case of presence of distant metastasis or in cases of solid carcinomas, micropapillary carcinomas or anaplastic carcinomas[126]. Ovariectomy at the time of surgical excision of the mammary tumor is seen critical as some studies conclude that there is neither a protective effect towards the development of new tumors and metastasis nor on the prognosis in the meanings of lifespan[126] yet other studies describe the contrary[130]. Practitioners should in any case perform a thoracic radiographic study in 3 projections to evaluate the presence of lung metastasis. In case of availability, computed tomography permits the diagnosis of metastasis of under 6 mm[131].

2.5.4. Pseudocyesis

Pseudocyesis or pseudopregnancy is a (para-)physiological event that manifests in the life of intact bitches. Clinical signs include nesting and maternal behavior, enlargement of the mammary gland and lactation[132]. Although part of a physiological process and often self-limiting, pseudopregnancy may present with important clinical signs which need treatment[133]. Depending on the severity of clinical signs veterinarians may opt for either no treatment or for pharmacological treatment, with the most commonly used drug being Cabergoline[133]. A relationship between pseudopregnancy and the development of mammary tumors has been discussed yet no relationship could have been proven[134]. Ovariectomy is the only preventive measure with permanent character[135,136]. Nevertheless, the timing of ovariectomy should be

carefully evaluated, as gonadectomy during lactation may result in persistent lactation[137] throughout life and neither should it be performed during diestrous (especially in bitches with history of pseudopregnancy) as the rapid change in hormonal concentration may initiate lactation[138,139].

2.5.5. Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is the most frequently found prostatic pathology in the elderly intact male dog[140,141]. The disorder consists in a benign enlargement of the prostatic gland[141]. Clinical signs include hematic discharge from the penis, urine dripping, difficulty in defecation and deformed feces[142,143]. Treatment is necessary in cases which present with clinical signs[141]. Treatment options for BPH include either pharmacological or surgical treatment[144]. The most commonly used drugs for BPH are Finasteride and Osaterone acetate, a molecule with potent anti-androgen effect[144]. In cases with frequent recurrence of clinical symptoms, orchiectomy may be performed[145]. The removal of the gonads results in a rapid decrease of 70% of prostatic dimension; a process that is notable already within the first 7-14 days after surgery and results in complete involution of the gland within 4 months[146,147]. Considering that more than 95% of dogs over the age of nine years are or will be affected by BPH, castration may be used also for prevention of this disorder[141,143,148]. Although often suspected, BPH has been shown to have no association with the development of prostatic neoplasia[149].

2.5.6. Testicular neoplasia

The testicles are the most common sites of urogenital neoplasia[123]. Most frequently found are Leydig cell tumor and Sertoli cell tumors[71,123]. Tumors within both genital and urinary tract have been reported to be found more frequently in the male rather than in the female[124]. The majority of testicular neoplasia found in the dog are benign[150]. Interestingly some testicular tumors may produce hormonal changes and may therefore cause signs of feminization[150]. Two of the most frequently found tumors originate either from Leydig cells or Sertoli cells and are denominated accordingly[151]. Leydig cell tumors are almost always benign and no reports are available on metastatic disease caused by this type of tumor[150]. Sertoli cell tumors instead are known to cause a feminization of the dog with signs such as gynecomastia, alopecia and hyperpigmentation, testicular atrophy and attractiveness for other male dogs[150,152]. It has been hypothesized that cryptorchid testicles are especially predisposed with intra-abdominal testicles being at highest risk for development[150]. Other possible types of testicular neoplasia are seminomas and teratomas[150]. Whereas testicular teratomas are rare[153,154], seminomas are

common tumors with an increased prevalence in inguinally retained testicles[150]. Due to changes in serum estradiol concentration, changes in the preputial smear may be present (similar to the vaginal smear of a bitch in heat) and used for diagnosis of the testicular tumor[155]. Considering that the majority of testicular tumors are benign, orchiectomy is considered a curative approach[156]. Nevertheless reports on aggressive testicular tumors exist[157]. Although orchiectomy may be performed as a curative approach in case of testicular neoplasia development, the advanced age of the individual at diagnosis[151] increases the anesthesiologic risks. Gonadectomy in males which are not destined for reproduction is preventive towards the neoplasia development and especially indicated in cases of cryptorchism.

2.6. Behavior and gonadectomy

Behavior and the effect of gonadectomy on it is a peculiar topic as it is not easily measurable and comparable and the impact of castration on behavior may be considered both beneficial and noxious, depending on the particular behavioral pattern in discussion. Although the impact of castration on various behavioral patterns of the dog have still to be investigated, owners frequently opt for surgery due to undesired/unwanted behavior of their dog. Prediction on how the surgery will affect the behavior of their pet in the long run is difficult and without consideration of a number of factors, nearly impossible. Behavioral patterns are multifactorially influenced and the importance of each factor may differ between individuals. Literature on the impact of gonadectomy in the dog is large yet inconsistent in its conclusions. Changes after castration and especially the possible impact of castration on aggression are aspects of utmost importance for the practitioner and the owner to allow an informed decision concerning gonadectomy of an individual.

The effect of castration on sexual behavior is documented and consists in a decreased response to the female (e.g. mounting, ejaculatory pattern)[158] also if male dogs have been reported to respond with a copulatory pattern up until 1 year after surgical castration[159].

In one investigation dogs were divided into two groups, one inexperienced (the males were allowed to mount but copulation was inhibited) and an experienced group (dogs were allowed to have 30 copulations prior to castration). Throughout the experiment after surgery, there were no differences in sexual behavior between the inexperienced and the experienced group[159].

Based on a retrospective clinic survey, owners reported a markedly reduced or completely eliminated objectionable behavior (including urine marking, mounting and intermale fighting in 50-60% of animals. Furthermore, owners reported a reduction or elimination of roaming in 90% of cases[160].

In females an increase in aggressive behavior towards the owner and indiscriminate eating has been reported, yet the increased aggression was apparent only in bitches which were castrated at less than 1 year of age and which already showed aggressive behavior at the time of gonadectomy[161].

In a Dutch study 124 dog owners have been interviewed concerning their orchiectomized dogs. Median age at gonadectomy of the males was 4 years. Behavioral problems were named most frequently as a reason to opt for orchiectomy, with undesirable sexual behavior being the most commonly named. Other described behavioral problems prior to castration include roaming, aggression (mainly towards other male dogs), fear and urinating both in- and outside the house. The owners reported that sexual behavior towards people decreased in 57% of the dogs[162]. Sexual behavior directed towards other dogs, male and female, diminished in 43% and 41%, respectively[162]. Impact on roaming was highly dependent on the motivating factor. Roaming due to olfactory stimulus (bitches in heat) reduced in 64% of cases, yet roaming behavior without an underlying sexual stimulus reduced only in 16% of cases[162]. Such a difference in effect of castration was observed also in aggressive behavior. Although aggressive behavior outside the house reduced in 52% of cases, such a reduction inside the house was observed only in 26%[162]. Fear was the only investigated behavior that remained unaffected by castration in the vast majority of cases [162].

An investigation of 1659 female and male dogs castrated at less than 1 year of age showed a statistically significant impact of castration on 6 of 7 investigated behaviors. “Urinating when frightened” was the only type of behavioral pattern which was not affect in a statistically significant manner [92].

Aggression, although often a cause for the owners request to castrate, is a behavioral problem of which a prediction of the effect of gonadectomy is very difficult. Results of studies are in disagreement with both reports on aggression being or not being affected by castration[163–165]. More importantly an increase in owner-directed aggression has been described in both females and males, an effect that appears to be breed-specific[161,165–167]. It appears that intermale aggression is the most controllable by gonadectomy in both dogs and cats[168,169] .

Behavioral problems were investigated in a population of service dog candidates. The population was composed of different breeds, with Labrador and Golden Retriever accounting for the majority. Behavioral problems that lead to dismissal from the training program include aggression, dominance, snapping or biting, growling, resource guarding, being confrontational, hyperactivity, fear and others. A total of 110 dogs has been dismissed from the training due to behavioral problems alone. In female and male dogs neutered at less than 7 months of age, 58%

and 42 % of dismissals were due to behavioral problems, respectively[90]. Of all females and males neutered between 7 and 11 months of age 33% and 35% of dismissals were due to behavioral problems, respectively[90]. In the group of animals neutered at more than 11 months of age, 54% of dismissals in females and 45% of dismissals in males were due to their inadequate behavior[90]. These results not only show that behavioral problems were the most frequent reason for dismissal but also that their occurrence were significantly less common in the group of dogs castrated between 7 and 11 months than in dogs castrated prior to 7 months or later than 11 months of age independently of the sex[90].

In 2016, an epidemiological analysis has shown that although castrated males and females are at a higher risk to develop a variety of behavioral problems, they are at a lower risk of developing problems related to aggression[170].

Stranger-directed aggression is a behavioral problem which may result in abuse, neglect and even euthanasia. It is therefore of importance to understand possible risk factors associated with this behavior. In an investigation including 14,310 dogs castration status was beside environment, owner experience and individual characteristic aspects, observed as a risk factor with castrated males being more likely to demonstrate aggression[171]. Sadly, any information on the time of castration is missing.

Most studies on behavior, as has been described for studies on negative health consequences of gonadectomy, divide their population either in early castrated, late castrated and intact dogs. Early castration may be defined either as castration prior to 12 months of age or may be further subdivided as has been described in the studies of Zlotnick et al[90]. Two studies on impact of castration on behavior instead did not only include a high number of animals (6,235 males[17]and 8,981 females[16]) but correlated the behavioral aspects with a calculated percentage of lifetime exposure to gonadal hormones (PLGH)[16,17]; an approach which allows in depth evaluation of the suspected impact and is nevertheless rarely used. The male dogs included in the study of McGreevy et al[17] have been castrated prior to 520 weeks of life due to reasons unrelated to behavioral problems. A total of 40 different behavioral patterns were investigated and have been grouped in *a.)* fear and anxiety (non-social fear, touch sensitivity, dog-directed fear, stranger-related fear, separation-related problems), *b.)* aggression (dog-directed, stranger-directed) and *c.)* excitability, energy and miscellaneous responses (excitability, energy, miscellaneous). Of the 40 included patterns, 25 have been associated with increasing PLGH whereas 14 were associated with a higher age at castration (AAC). Of these 25, 23 patterns were reduced and 2 (howling and marking) were increased. Regarding behavioral patterns associated with AAC 13 were reduced and marking as the only pattern increased. Most

importantly 8 and 7 of the behavioral patterns that decreased with increasing PLGH related to fear and aggression, respectively[17]. The methods of evaluation of the population of 8,981 female dogs did not differ from the evaluation of the male population. Of 23 behavioral patterns 12 were associated with increased PLGH and 5 with higher AAC. Although chewing and howling were seen significantly more frequently in females with longer PLGH 10 patterns (1 related to fear, 3 related to aggression) were significantly reduced with increased PLGH. Concerning increased AAC 3 behavioral patterns were significantly decreased, 4 were related to excitability, energy and miscellaneous responses whereas 1 was related to aggression[16]. On the basis of these results it may be hypothesized that although behavior may be influenced by a wide range of factors (e.g. socialization, handling, degree of training), the passage from puppy to adult through puberty and thereby the effect of sexual hormones has a positive effect on behavioral patterns related to fear and aggression.

Prediction of effects of gonadectomy on the behavior of a dog is of special importance in the case of animals destined to be a working dog (e.g. police dogs, military dogs, service dogs). Especially for police and military dogs reactivity is a factor of utmost importance, which is evaluated using a scoring system[172]. For the investigation of the effect of castration on reactivity a population of German Shepherd dogs were used, which were gonadectomized. On the contrary of the belief that castrated animals become calmer, the bitches within this study showed an increased reactivity compared to their intact peers[172]. It remains to be evaluated if this effect is associated to breed or not.

Although the above-mentioned studies differ in their results and conclusion on how castration impacts aggression (increase or decrease), all of them nevertheless agree that gonadectomy has an impact. In 2016 on the other hand Farhoody et al[173] published the results of their experimental study on the impact of gonadectomy on aggression in the dog using a very large population of dogs. These authors conclude that gonadectomy itself and the age at gonadectomy have no significant relationship with aggressive behavior towards familiar people, strangers or dogs[173].

An important factor of canine behavior for the society rather than the pet owner is the occurrence and prevalence of dog bites. Especially in countries with a high number of stray dogs, dog bites and dog attacks are a problem. Castration is used in stray populations to reduce uncontrolled reproduction and the impact of castration on the number of dog bites has been investigated. Although results and conclusions on the impact of castration on aggression are not completely consistent, the literature suggests, that castrated dogs roam less and that the

number of dog bites decreases[174]. It has to be considered nevertheless that behavior resulting in dog bites is multifactorial.

2.7. Conclusion

Taken all considerations and results of studies into consideration, there is still a lot we don't know on the impact of gonadectomy on the canine organism. Furthermore, the limitations of studies as described within this summary and the published review on prostate carcinoma (Appendix 2) give reason to conclude, that we know far too little to be able to take a decision for or against elective gonadectomy. Each case has to be evaluated using a thorough clinical history including the behavioral patterns of the animal and possibilities of management of the owner prior to decide if the subject is a valid candidate for surgical castration. Nevertheless, the owner needs to be informed, that our current knowledge doesn't permit to predict the effects especially long-term effects with absolute security. It may be useful to create a list of possible beneficial and noxious long-term effects for each individual and to rank them depending on the incidence found within studies for each effect. The protective effect of gonadectomy towards pyometra and BPH which are both disorders with a high incidence, may be considered more important than a possible, but not yet proven effect on the development of prostatic neoplasia, which has a very low incidence within the canine population. Until further research is available, there will remain some doubt for both the practitioner and the owner.

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3. Immunohistochemical evaluation of steroid hormone receptor expression in extra-genital tissue in the bitch motivated by reports on negative long-term health effects of gonadectomy

3.1. Steroid hormones and their effect in the organism:

The group of steroid hormones may be divided into two classes based on their site of production (corticosteroid and sex steroids) or into 5 groups depending on their receptors (glucocorticoids, mineralocorticoids, androgens, estrogens and progesterone). The present introduction to the experiment and its preliminary results will be concentrating on sex steroids (androgens, estrogens and progesterone).

The effect of sex steroids on reproduction has been investigated in depth, yet there is still a lot to understand about the effect of these hormones on extragenital tissue. Especially reports of increased risk for development of pathologies in these extragenital tissues make an improvement of this knowledge important.

3.1.1. Bone

In the human, osteoporosis is a common disorder resulting in an increased fragility of the bone[1]. The increased fragility may result in pathological fractures of the bone and are an important cause of not only morbidity but also mortality in the elderly human[2,3]. Especially women experience an increased speed of bone loss after the menopause[4–7]. Estrogen deficiency is therefore considered an important pathogenetic factor in postmenopausal osteoporosis[8]. Fewer is known about the effect of declining testosterone concentration on the bone. A study using 40 male adult rats has shown that number of osteoclasts significantly increased in castrated animals compared to their intact peers[9]. Considering these reports, a similar interaction between steroid hormones in the canine species may be hypothesized yet up until today no empirical data is available on the effect of steroid hormone deficiency on the canine bone. Furthermore, it has to be noted that the primary negative effect of steroid hormone deficiency is associated with a generalized decreased bone density whereas the suspected side effect in the dog is a neoplastic disorder, which has to be taken into consideration when attempting comparison with the human.

3.1.2. Cardiovascular system

In the human a significant sex difference in cardiovascular disease has been reported with men being at a higher risk compared to premenopausal women. This difference changes once women pass the menopause which results in an increase in mortality in postmenopausal women exceeding the rate of men[10–14]. A beneficial role of estrogen on the vascular system is therefore presumed by many studies[15]. Treatment with estrogens has been shown to have a beneficial effect on heart failure in mice through binding on estrogen receptor beta (ERβ). Furthermore, binding to this receptor has been linked to increase cardiac angiogenesis, restore of hemodynamic parameters and suppress fibrosis[16].

3.1.3. Other organ systems

The influence of ovarian hormones on the involution of the thymus is well-known[17]. As in humans, also in rats, the involution of the thymus starts after puberty. An experimental study using prepubertal ovariectomized rats compared to intact rats of the same age. Evaluation of T-cell production showed a significantly higher number of T-cells in the group of castrated animals compared to the group of intact ones[18]. Changes in size and composition of T-cells may influence the animal's risk for immunologically-mediated pathologies[19–21], which makes this finding particularly interesting. The effect of estrogen on T-cells has further been confirmed by an in vitro study. Splenic lymphocytes of rats have been treated with different concentrations of 17β-estradiol which resulted in an upregulation of the T-cell immune response at low concentrations of the agent[22].

In mice estrogens have been further linked to changes in the hematopoietic system. Findings have shown that hematopoietic stem cells divided significantly more frequently in females than in males with an additional increase during pregnancy. Furthermore, erythropoiesis in the spleen was increased in females compared to males with an additional increase during pregnancy. To prove an association between this phenomenon and estrogens, estrogen was administered to male mice resulted in an increase of stem cell division also in males[23].

In the dog serum testosterone levels have been linked to medically induced gingival hyperplasia. The problematic occurs in humans which are in treatment with calcium channel antagonists. In a dog model, gingival hyperplasia was induced and the effect of serum testosterone levels were investigated, showing that there is a significant positive correlation between gingival hyperplasia and testosterone which leads to the hypothesis that testosterone may be a useful molecule for treatment of secondary gingival hyperplasia in the human[24].

The brain although being partly responsible for maintaining the reproductive cycle, it is nevertheless also target of sex steroid. Studies have shown, that steroid hormones may have a stimulatory effect on brain cells, (e.g. estradiol may lead to an increase of dendritic spines of neurons) and on the expression of receptors within the brain (estradiol and progesterone may increase the number of oxytocin receptors)[25].

Other organs which have been reported to be influenced by estrogens in the human are the liver[26,27], the intestine[28,29], the spleen[30,31] and the kidney[32] with the presence of estrogen receptor α (ERα) proven in liver, spleen and intestine and the presence of ERβ in intestine and kidney[33].

3.2. Current knowledge on steroid hormone receptor expression in different species including the dog

Steroid hormone expression has been evaluated in a number of species using different methods (e.g. PCR, Western blot, immunohistochemistry). Literature on steroid hormone receptor expression in urogenital tissue is vast[34–36] whereas the number of studies performed using extra-genital tissues is much less and is of main interest for the present experiment.

In the rat estrogen receptor expression has been investigated in cardiovascular tissue (heart, aorta, kidney and adrenal gland), the brain (somatosensory cortex, hippocampus and prefrontal cortex) and reproductive tissue (ovaries, mammary gland, uterus and testes) using PCR. Within tissues considered as cardiovascular ERα was significantly more present than other receptors in the adrenal gland, the aorta, the heart and the kidney in both male and female individuals. The same may be said about the results of mammary tissue and uterus. Concerning the three brain areas the G-protein coupled estrogen receptor (GPER) was the most commonly found. Estrogen receptor β was found yet in all tissue types significantly less than ERα and GPER[37].

Regarding the role of sex dimorphism, a study reports ERβ expression to not different between males and females, whereas ERα expression has been reported to differ dependent on sex[38].

Heart tissue has been evaluated also in mice. Through the use of Western blot analysis, the expression of ERα, ERβ and androgen receptor could be proven in atrial and ventricular tissue in both males and females. An interesting finding remains that steroid hormone receptor expression did not differ between ovariectomized and sham-operated mice[39].

In the human, the presence of ERα and ERβ have been proven in cardiomyocytes[40], cardiac fibroblasts[41] and in vascular smooth muscle cells (VSMCs)[42], yet inconsistencies in previous reports leave many questions open regarding both the receptors expression and its cellular

locations[43]. ERα has been reported to be present equally in men and women[40,44], whereas ERβ reportedly have a greater expression in men[45].

Also, canine vascular tissue has been attributed to be targeted by androgens, estrogen and progestins. Mixed breed males and intact and castrated females have been dissected after death and samples of the aorta and the inferior vena cava have been taken. Through hormone receptor assays, the presence of estrogen and androgen receptors in the aortic samples of intact animals has been confirmed. Steroid hormone receptors in the vena cava of bitches instead have been found to be reduced to 20% of the levels found in the aorta. In the samples of the vena cava in males, all steroid hormone receptors have been completely absent. Progesterone receptors (PR) showed to be peculiar in the sense that their presence appears highly legated to the presence or absence of estrogens. In the aorta of spayed animals, PR were absent. To further evaluate the association between estrogens and the presence of PR, castrated bitches have been injected with estradiol-17β for 1 week. In bitches dissected after this substitution treatment PR were present in the tissue sample of the aorta[46].

To the present day and to my best knowledge the mast cell tumor is the neoplasia extra-genital tissue associated with castration in which the presence of sex steroid hormone receptors was confirmed. Although the sample size includes tumors of only two individuals, the radio-receptor assay could confirm the presence of estrogen receptors (in both cases), and the presence of progesterone receptors in one case[47]. The skin was the subject of another investigation which attempted to explain a possible correlation between pathologies and castration. On the contrary to the idea that steroid hormones may be the most important initiating factor, these authors used both immunohistochemistry and Real-Time PCR to evaluate gonadotropin releasing hormone (GnRH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) receptor expression. Samples were taken from 10 euthanized dogs between 4 and 11 years of age. All three receptors were expressed in the skin of the dogs[48]. To further evaluate the impact of these results towards a possible solution on the pathoetiology, of neoplastic side effects of castration, it appears useful to compare the results with a population with information on sex and neuter status.

The negative effects on health apparently caused by gonadectomy in the dog is the motivation for both in depth research and the development of the in the following described experiment. To determine the presence or absence of estrogen and progesterone receptors in the tissues of origin of castration-related neoplastic disorders is of importance regardless the results. In case of receptor expression within these tissues future research will be needed to understand how

the hormones may impact the physiology of these cells. In case of negative results, the approach of Welle and coauthors[48] may be applied for the evaluation of GnRH, FSH and LH receptors.

3.3. Bibliography

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4. Immunohistochemical investigation of the expression of estrogen and progesterone hormone receptors in extra-genital tissue in the female dog: preliminary results

4.1. Introduction

Research on hormone receptor expression has become a very important aspect in both human and veterinary medicine. Especially in oncology determination of hormone receptor expression has become a method of diagnosis, and may provide very useful information contributing to the decision for the right treatment in human medicine[1–3]. In the case of the human breast cancer, the expression of estrogen (ER) and progesterone receptors (PR) are routinely evaluated in biopsy samples and are considered both prognostic and predictive markers[4]. Advances have been made in veterinary medicine as well, yet techniques and investigations concerning hormone receptor expression are not routinely used for diagnostic purposes[5,6].

Over the last two decades reports on negative long-term health effects of gonadectomy on the male and female dog have increased. Reports on the effect of gonadectomy on the risk of tumor development increased yet no causal relationship could be proven so far. An increased risk for castrated individuals has been reported for lymphoma[7], prostate carcinoma[8], mastocytoma (MCT)[9], haemangiosarcoma[10] and osteosarcoma[11,12]. As early as in 1982 estrogen and progesterone receptors have been identified in the tissue of a mast cell tumor of a dog. These authors describe growth inhibition and cell death as an effect of steroid hormones[13]. Further an interaction between bone tissue and steroid hormones has been investigated in the human due to high incidence of osteoporosis and its increased incidence in women after the menopause[14]. Although data on steroid hormone receptor expression in canine extra-genital tissue is scarce, immunohistochemistry (IHC) has been used to evaluate the receptor expression of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the skin and the urinary bladder of dogs [15]. Although a positive result on the presence of certain hormone receptors does not explain how this hormone might affect the tissue, it gives the possibility to determine if a certain hormone might have a direct effect on it. Nevertheless, secondary pathways may be possible and should not be excluded from the list of possibilities. Intense research over a long period of time will be necessary to determine and prove a causal relationship between the absence of sexual hormones and the development of malignant neoplasia in non-reproductive tissue in the dog. The aim of the present study was to investigate the possibility of the most direct possible pathway of the presence or absence of

steroid hormones on the heart, spleen and lymph nodes in the dog, by investigating the presence or absence of steroid hormone receptors.

4.2. Materials and Methods

4.2.1. Population

A total of 14 bitches have been included in the postmortem collection of heart, spleen and lymphatic tissue (see Table 1). These bitches were admitted and treated in the Veterinary Teaching Hospital of the University of Padua between November 2016 and July 2018. Age at death ranged from 2.1 to 16.7 years. Ten bitches were castrated at the time of death, yet age at castration was available only in 4 cases. Two bitches have been castrated at less than a year of age, one bitch at 7 years and the fourth one at 15.25 years. Type of death was recorded with 4 bitches dying spontaneously whereas 10 bitches have been euthanatized due to bad general condition and poor prognosis. Bitches have been included in the study population when adequate information on anamnesis, signalment, motive of death and type of death was available. Furthermore, inclusion into the study was possible only after owners' consent.

Table 1: Overview of the studied population

Subjects	Age at death (years)	Breed	Sex status	Type of death
1	5.9	Mixed breed	Castrated	Euthanasia
2	2.1	Rottweiler	Intact	Spontaneous
3	7.4	German Shepherd	Intact	Euthanasia
4	12.2	Mixed breed	Castrated	Euthanasia
5	8.4	Yorkshire Terrier	Castrated	Spontaneous
6	2.5	American Akita	Castrated	Euthanasia
7	16.7	Epagneul Breton	Castrated	Euthanasia
8	12.2	West Highland White Terrier	Intact	Euthanasia
9	13.0	Mixed breed	Castrated	Euthanasia
10	7.1	Mixed breed	Castrated	Euthanasia
11	14.1	Mixed breed	Castrated	Euthanasia
12	16.0	Mixed breed	Castrated	Spontaneous
13	11.9	Mixed breed	Castrated	Euthanasia
14	4.4	Dobermann	Intact	spontaneous

4.2.2. Tissue sampling

After clinical diagnosis of death (asystole, apnea, absence of reflexes) the corpses were prepared by clipping of the abdomen and thorax, the retromandibular and the popliteal region. The corpse was then positioned in dorsal recumbency to allow easy access to the above-mentioned regions. Prior to incision the skin was scrubbed with Chlorhexidine solution. The order of tissue sampling remained unchanged for all animals throughout the study period. Tissues were sampled in the following order: popliteal lymph node, mandibular lymph node, right ventricle of the heart, spleen. Both popliteal and mandibular lymph nodes were sampled in toto bilaterally. After a midline incision reaching from the manubrium of the sternum to the middle of the abdomen the heart was removed in toto through a thoracotomy after clamping of the vena cava and the aorta. The pericardium was opened and a piece of 2x2 cm of the right ventricular wall was removed. The spleen was accessed by a cranial midline laparotomy and a piece of at least 2 cm of length and 1 cm of width of the splenic hilum has been removed. A second splenic sample was taken from one of the two splenic extremities. In case of presence of neoplasia in either of the tissues of interest, a sample was taken of the pathological areas as well.

4.2.3. Tissue preparation and histological staining

Tissue samples (Heart, Spleen and Lymph nodes) were collected during necropsy, within a maximum of 4 hours after death. Then, samples were fixed in 4% neutral-buffered formaldehyde (NBF) to prevent tissue autolysis. After fixation, tissues were dehydrated by immersion in increasing concentrations of alcohol to facilitate embedding with paraffin. Blocks of paraffin-embedded tissue were then sectioned in 4 μm adjacent sections using a Leica RM 2145 microtome. For histological observations, sections were stained with hematoxylin and eosin (H&E) following a routine protocol [16]. To summarize, the sections were deparaffinized in xylene solutions and after in decreasing concentration of alcohol solutions for hydration of the tissue. The sections were then rinsed in hematoxylin for about 10 min and after in eosin for 30 seconds. Finally, sections were dehydrated in alcohol and xylene and routinely mounted in resin (Eukitt mounting medium; Electron Microscopic Services, Fort Washington, Pennsylvania).

4.2.4. Immunohistochemistry

The expression of the following antigens was evaluated with anti-human antibodies (Abs): monoclonal rabbit progesterone receptor and monoclonal mouse estrogen receptor.

Staining for immunohistochemical analysis was executed by an automatic immunostainer (Ventana BenchMark GX, Roche Diagnostic), which employs a kit with a secondary antibody and a horseradish peroxidase (HRP)-conjugated polymer that binds both mouse and rabbit primary antibodies, antigen detection with ultraView Universal DAB kit (Ventana Medical Systems) and counterstaining with hematoxylin. All the reagents were dispensed automatically with exception of the primary antibody, which was applied manually. Finally, slides were manually dehydrated through a graded series of alcohols and mounted (Eukitt mounting medium; Electron Microscopic Services, Fort Washington, Pennsylvania). ER dilutions were performed using a commercial antibody diluent (Ventana Medical Systems), while PR were ready to use. Specific antibodies characteristic, dilution, time and temperature of incubation are stated in Table 2.

Dog uterine and ovary section was used as control to confirm the cross reactivity of the Abs with canine tissues and the test performance. Another control (the blank) was performed by applying the secondary Ab without the primary Ab, in order to confirm that there was no reaction between the secondary Ab and the tissue.

Table 2. Detailed information of the Abs used for the immunohistochemical analysis

Antigen	**Features**	**Clone**	**Dilution**	**Incubation of primary Ab – Temperature (°C) and Time (min)**	**Localization**
Progesterone receptor	Ventana; Monoclonal rabbit anti-human	1E2	Ready to use	37°C for 32 min	Nuclear
Estrogen receptor Alpha	Novocastra; Monoclonal mouse anti-human	EP1	1:40	37°C for 32 min	Nuclear

4.3. Results

4 dogs involved in the study died spontaneously, while 10 were humanely euthanized in the Veterinary Teaching Hospital of the University of Padua.

The studied population is composed by a variety of breed with dogs of mixed breed seen most frequently. Sexual status was evaluated at the time of death. Age at gonadectomy was known in 4 of the 10 castrated animals. Of these 4 bitches, two have been gonadectomized at less than

a year of age on an elective basis, whereas two bitches have been castrated at 7 and 15 years of age respectively due to uterine pathologies.

Histologically, all tissues sampled were analyzed and a precise description was made for each slide. The organs selected were normal o mildly compromised (mild diffuse congestion or mild multifocal cellular depletion in spleen and mild multifocal presence of brownish granular pigment in cellular cytoplasm in lymph node) in 11 dogs, while 3 dogs presented neoplasia (2 in heart and 1 in spleen). Location and histological image are consistent with the presence of cardiac or splenic hemangiosarcoma in the cases of these 3 bitches.

As expected, the blank control resulted negative, while the two antibodies positively reacted with dog control tissues (positive control). In fact, the IHC showed strong positive nuclear staining using both ERα and PR antibodies (Figure 1 and 2, respectively). On the other hand, investigation for ERα and PR showed negativity in all the tissues selected including both physiological extra-genital tissue (Figure 3 and 4) as well as samples from the three neoplastic alterations.

Fig. 1. Estrogen receptor immunohistochemical labelling of selected canine control tissue (uterus). The majority of epithelial cells labelled positively for estrogen receptor (nucleus). Mayer hematoxylin counterstained; original magnification 40X.

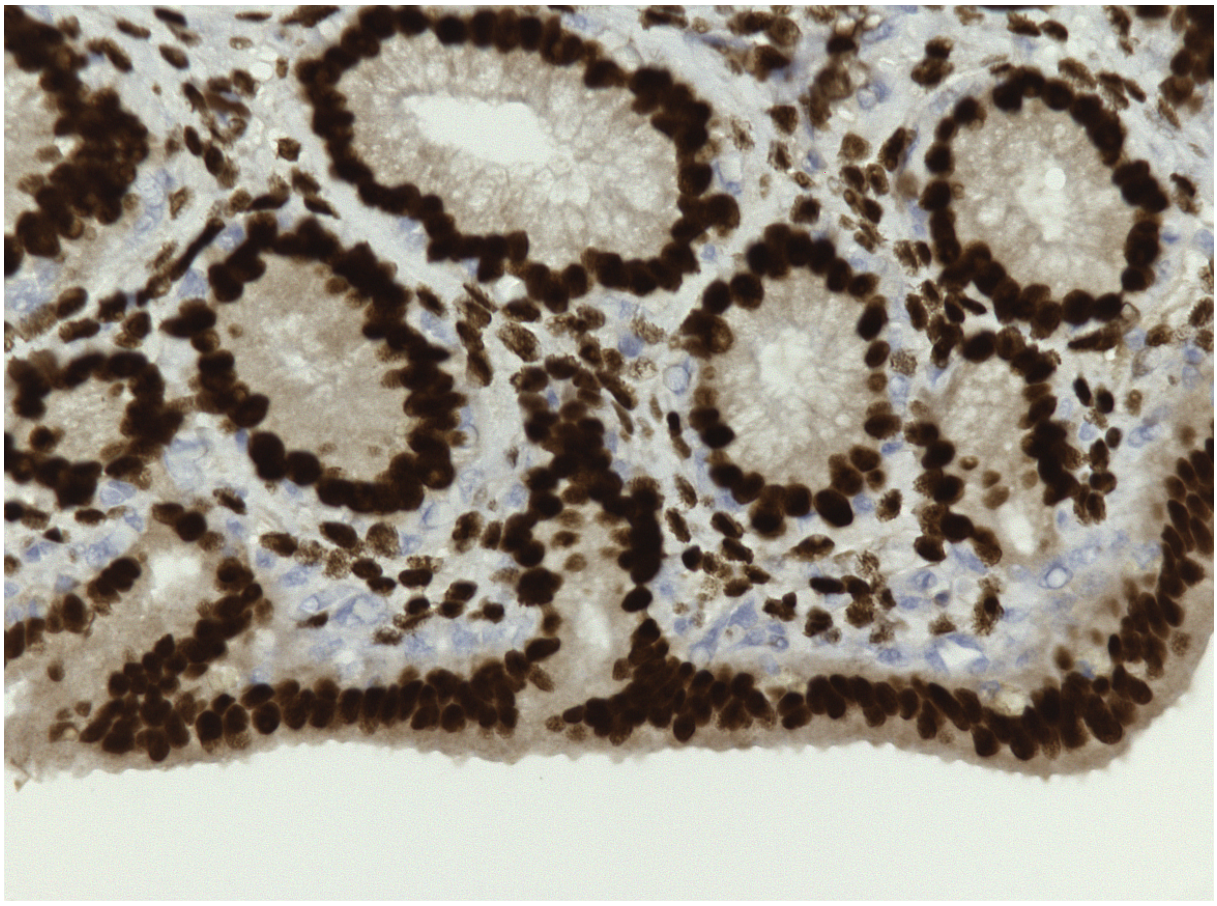


Fig. 2. Progesterone receptor immunohistochemical labelling of selected canine control tissue (uterus). The majority of epithelial cells labelled positively for estrogen receptor (nucleus). Mayer hematoxylin counterstained; original magnification 40X.

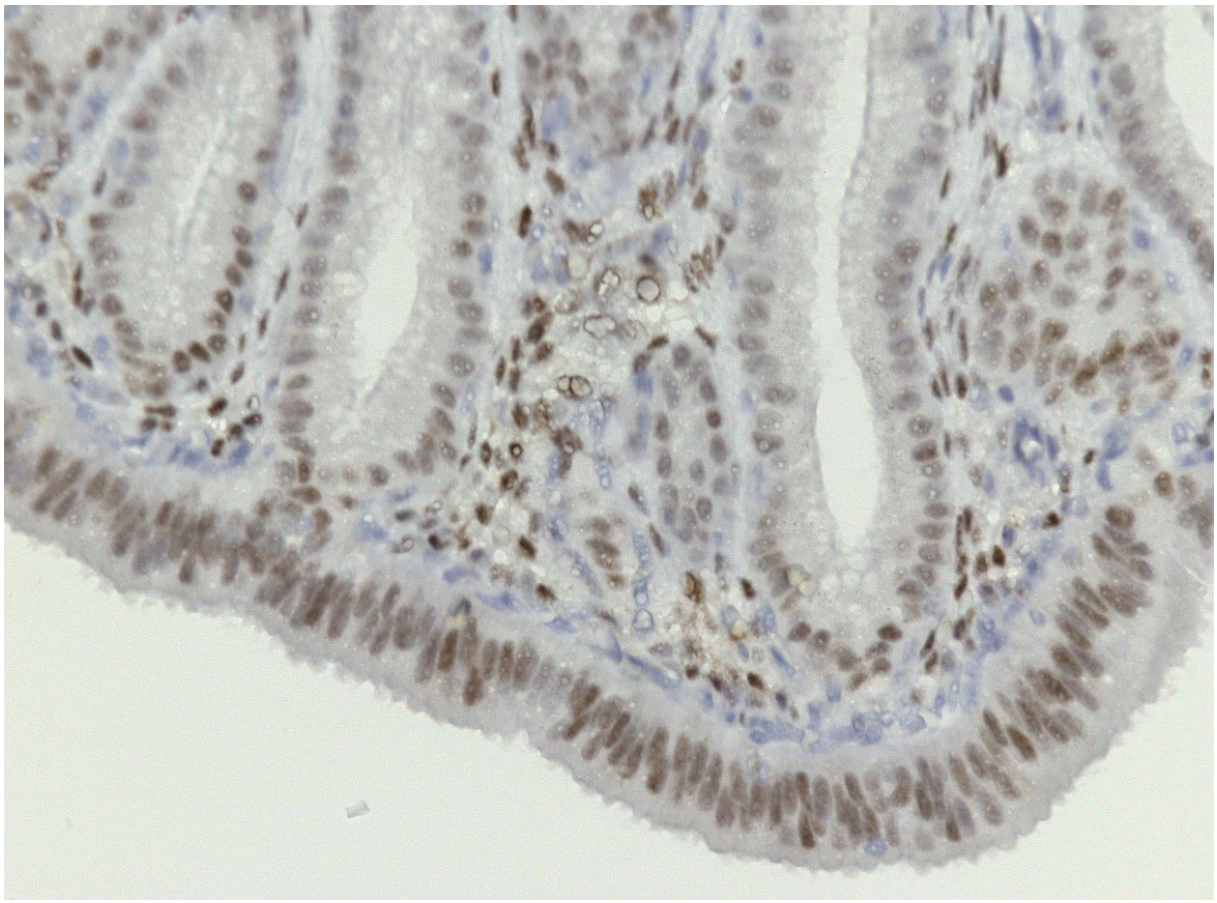


Fig. 3 (a and b). Estrogen receptor immunohistochemical labelling of canine cardiac muscle. All cells did not react for estrogen receptor. Mayer hematoxylin counterstained; original magnification 40X.

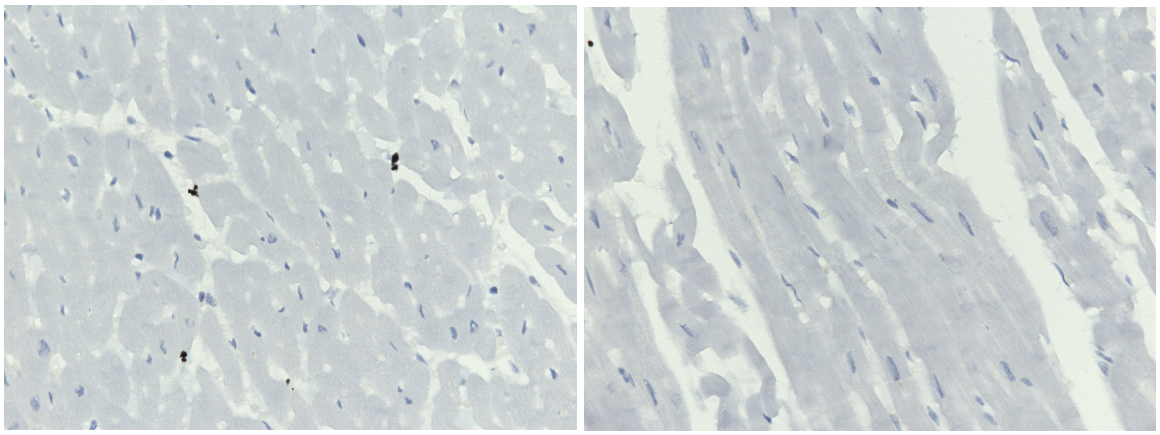
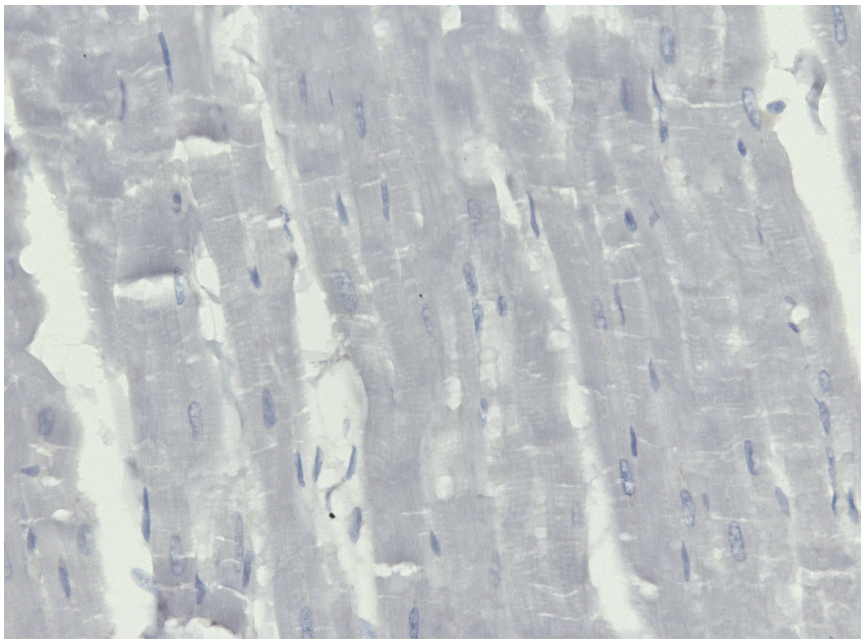


Fig. 4. Progesterone receptor immunohistochemical labelling of canine cardiac muscle. All cells did not react for estrogen receptor. Mayer hematoxylin counterstained; original magnification 40X.



4.4. Discussion

4.4.1. Method

Immunohistochemistry is a widely accepted method used for evaluation of hormone receptor expression in various tissues and species. Especially its ability to not only provide information on presence or absence of the researched receptors but also on its quantity and position within the tissue cells is most appreciated and gives the possibility to increase understanding on possible pathways and future treatment options[1,3].

4.4.2. Motivation for the study and implications for the future

Over the last decades the use of this method allowed important improvements in the understanding and treatment of various neoplastic disorders in the human and other mammals such as the dog. Disorders in the human such as lymphoma[7,17], osteoporosis[18] and others are known to be more frequently found in women after the menopause. This peculiar aspect of these disorders creates the hypothesis that changes in the concentration of sexual hormones within an organism may have important implications on extra-genital tissue. It is therefore that the effects of steroid hormones on lymphatic and bone tissue have been evaluated in depth using various different methods. The presence of steroid hormone receptors within a tissue may be

considered a prove of a direct effect of these hormones on the tissue, although its absence may not exclude an effect as secondary pathways may be present.

The population of domestic dogs presents a population which is used as an animal model for various disorders of the human (e.g. prostatic carcinoma). Especially the availability of both intact and gonadectomized animals combined with similarities in pathoetiology, and progression of diseases makes the dog a very valuable species for animal models[19–22]. Although different studies have been motivated to provide insight in disorders of the human, advances in veterinary medicine provided the basis to investigate disorders in the canine species not only for the benefit of the human but for the benefit of pet dogs as well.

Gonadectomy is still the most commonly performed elective surgery in the canine species, mainly to provide control of unwanted reproduction. Especially prior to 2000, gonadectomy has also been advertised to be a useful tool to control undesired or problematic behavior and was considered an intervention with a beneficial effect on the animals' health and well-being. This assumption of a low risk surgery combined with no negative side effects in the long-term changed as since the beginning of the century more and more reports surfaced which linked gonadectomy in botch bitches and males to serious long-term effects on the animals' health. Although not all of these reports are to be considered valuable due to important differences and shortcomings in the study design, the presence and numerosity of these reports created doubt and concern within the group of practitioners and owners. A negative effect in the meanings of an increased risk for the development of various neoplastic disorders in extra-genital tissue has been reported using statistical methods yet until today no hypothesis on the possible causal relationship has been established and proven. Changes within the organism of a gonadectomized dog include the absence of estrogens, progesterone and/or testosterone, yet with a remaining low concentration of hypophyseal hormones such as LH and FSH. Research on the presence or absence of hormone receptors within extra-genital tissue has been performed in the mouse and the rat for all these hormones, yet no such study is available concerning the hormone receptor expression for progesterone and estrogen within the canine organism. We decided therefore to begin our investigation with the research or PR and ERα as the changes of these hormones are faster and more important compared to LH and FSH when considering the time after castration.

Canine urinary bladder and skin tissue have been proven to express receptors for GnRH, LH and FSH. The authors of this study consider the possibility that the decrease of hypophyseal hormones may be the cause for the development of reported side effects of castration rather than being a consequence of the change in estrogen and progesterone concentration[15].

The role of estrogens and its effects on the cardiovascular system have been investigated in different species. In 2009 the presence of estrogen receptor α, estrogen receptor beta and androgen receptor has been proven in mouse heart by Western Blot[23]. The study included tissue samples of cardiac atrium and ventricle of male and female mice. Interestingly, ovariectomy of the mice resulted in a significant increase in ERα within the ventricle. Although the expression and distribution of these receptors did not appear to be sex related, the results suggest a chamber specific expression of ERα[23]. Canine vascular tissue has been investigated using a receptor essay and the presence of ER is reported in vascular tissue such as the aorta.

4.4.3. Composition of the Population

The animals enrolled in the study were both castrated and intact bitches of various ages. This heterogeneity of the population gives the possibility to diminish a possible effect of age, sexual status or breed on the results. Although the number of animals enrolled may be considered low, the number of samples (four different tissues per subject) may be considered enough to give reason for further research in this area. Sadly, information on the date or age of the animal at castration was available only in 4 of 10 subjects. This information may be considered of prime importance and should be included in the composition of future study populations.

4.4.4. Sampling

The decision on which samples to include in the study was based on the current literature. Lymphoma and hemangiosarcoma are only two of four neoplastic disorders in extra-genital tissue in the dog linked to an increased risk in castrated animals. Canine hemangiosarcomas are known to develop mostly out of vascular, cardiac and splenic tissue. Due to this the samples were taken from areas in which both vascular and parenchymal tissue could be evaluated, namely the right ventricle with coronary vessels and the splenic hilum. Concerning the lymphatic tissue, sampling of the retromandibular and popliteal lymph nodes as example for lymphatic tissue was based on considerations of ease of access but also on the fact that these are lymph nodes which are evaluated in every clinical exam and therefore one of the first indicators of a possible lymphatic pathology. The retromandibular lymph node, due to its location, may be found with alterations rather frequently as inflammation or infection of the oral cavity, teeth and ears affect it. The popliteal lymph nodes instead are rather distant from bacterially charged organs and are mostly reactive in case of generalized or systematic pathologies/disorders. To be able to conclude the absence of ERα and PR for the lymph nodes,

samples should be taken also from other sites such as abdominal and other less easily explorable lymph nodes.

4.4.5. Handling and Preparation

Sampling, handling, conservation and preparation of the tissue samples were performed as has been reported previously for other immunohistochemical studies [24]. Fixation in 4% neutral-buffered formaldehyde (NBF) has been considered the gold standard in surgical pathology for decades, also if research on a viable substitute continues due to its carcinogenic characteristics[25]. Formaldehyde is cheap, a good preserver of morphological features and allows long-term storage[25]. Although the agent is considered to allow reliable immunohistochemical analysis, a risk of cross-linking which masks antigens is reported to create a possible bias for IHC[26,27]. We therefore assume that our negative results may hardly be caused by any of these steps. To completely exclude a possible bias cause by handling or conservation future samples could be preserved in glyoxal or non-cross-linking alcohol-based fixatives. These agents are inferior in their performance for immunohistochemistry yet for most antibodies staining worked well enough. Furthermore, with agents like FineFIX/RCL2 NBF-based protocols may be maintained yet without the risk of carcinogenic vapor. Nevertheless, a change in fixation agent requires a validation of the protocols and reevaluation of the markers used, as their sensitivity and specificity was tested using NBF[25]. Glyoxal has been reported to be superior in its fixation capabilities compared to NBF and showed good results in immunostaining of various tissues[28]. According to the guidelines of the College of American Pathologists, samples of human breast tissue must be fixed for at least 6 hours in 10% neutral-buffered formalin prior to elaboration for the research of PR and ER[3]. Prolonged fixation (>72 hours) of human breast tissue has been reported to possibly lead to a reduced immunoreactivity and creates therefore the risk of false-negative results in the research of hormone receptor expression [29,30]. On the contrary it has been reported in 2012, that there were no significant qualitative changes concerning the results of ER and PR in human breast tissue preserved for 96 hours compared to those preserved for less than 72 hours[4].

4.4.6. Antibodies

Antibodies validated and advertised for a specific species and are often difficult to find and are not always available. The validity of the antibodies used in the present study, although not assured by the company, was tested and proven. The positive control used for both PR and ERα was canine uterus and canine ovary. Both tissues are known to express both receptors and were

chosen due to this. The samples of ovary and uterus were handled and preserved in the same way, although the sampling took place during an elective gonadectomy and may therefore be considered to have taken place and much stricter sterility of the environment and instruments than the postmortem sampling. A break of sterility during the sampling process should not have had an impact on the results as the method of immunohistochemistry does not rely on protein expression as does for example the Western Blot but depends on the binding of the antibody to the expressed receptor. Both antibodies used in this study reacted positively with canine uterine and ovarian tissue and we therefor considered them useful for the research of PR and ERα in extra-genital tissue. The negative control instead assured that no reaction between the tissue and the secondary antibody was present. Therefor any bias resulting from the antibodies may be excluded.

4.5. Conclusion and considerations for the future

Discussion on the usefulness of elective gonadectomy in the dog and the possible fatal health risks increased within the professional community. Pet owners have now, as they never had before the possibility to inform themselves autonomously over the internet. This unguided research for information which is often presented completely out of context results in the increased doubt towards elective gonadectomy. We consider our result preliminary yet a first step after which more research needs to follow. It seems naïve to believe that the removal of an endocrine organ from an organism has no side-effects on other organ systems within this organism. Nevertheless, prove is needed to rightfully exclude elective gonadectomy from the daily veterinary practice and other strategies must be evaluated to avoid uncontrolled reproduction of both stray and pet dogs. The negative results of the present investigation have to be supported by a second laboratory test, so a Western Blot will be performed in the near future. In case of confirmed absence of PR and ERα within the studied tissues receptors for LH and FSH may become of primary interest. In case of positive results for the expression of PR and ERα in the Western Blot, the immunohistochemical evaluation should be repeated with changes in handling and most importantly conservation of the tissue samples.

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5. APPENDIX

5.1. APPENDIX 1

animals

MDPI

Article

Bodyweight at Birth and Growth Rate during the Neonatal Period in Three Canine Breeds

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Simple Summary: The 349 recognized canine breeds differ greatly in bodyweight and, therefore, in birthweight and neonatal growth. The weight and growth of puppies are easily measurable, and are possible early indicators of problems. Low birthweight has been linked to neonatal mortality based on results obtained by grouping breeds according to their adult bodyweight. Breed-specific ranges of birthweight and growth would allow for the identification of puppies at risk. Our aim was to evaluate the birthweight and early growth of healthy puppies of three breeds in a breed-specific manner. Birthweight, expressed as percentage of mothers' bodyweight, showed that puppies of a large breed are born smaller than puppies of a small breed. Puppies of a large breed gain weight slower than puppies of a small breed. Sex has no impact on birthweight, whereas litter size influences birthweight and weight gain. Based on our procedure, we considered 29 of 213 puppies to be of a low birthweight, whereas 160 of 213 might have been considered of a low birthweight if using the classical criteria (based on breed groups). This shows the importance of breed-specific evaluations of birthweight. Further research is needed on the importance of breed-specific evaluations for early growth.

Abstract: Weight at birth (bBW) and early weight gain have been linked to the risk of neonatal mortality. Pups are described to be of low bBW if weighing less than one standard deviation (SD) below the mean. Most studies classified breeds according to their expected adult bodyweight. Our aim was to evaluate the breed specificity of these parameters. We assessed the bBW of 213 puppies of Bernese Mountain Dog (BMD), Tibetan Terrier (TT), and Lhasa Apso (LA) breeds, as well as the neonatal growth rate of 133 puppies of BMD and TT. BMD puppies were born relatively smaller than puppies of TT and LA ($p \leq 0.0001$) and gained less weight than TT puppies during the first 14 days ($p \leq 0.05$). Litter size had a significant impact on bBW and daily gain until the onset of the third week for BMD ($p < 0.0001$ and $p = 0.0005$, respectively) and TT ($p = 0.0003$ and $p = 0.0064$, respectively). When using bBW means and SD specifically assessed according to breed, 29 out of the 213 neonates of our study were judged as being of low bBW, whereas, when using the classical criteria (based on breed groups), the number of low bBW pups was 160 of 213. These results suggest that evaluations of bBW and neonatal growth should be performed in a breed-specific manner.

Keywords: puppy; birthweight; neonatal growth; litter size; daily gain

1. Introduction

The domestic dog represents a species with a wide variability in phenotypical appearance due to the abundance of its breeds. The Fédération Cynologique Internationale (FCI) recognizes 349 breeds, ranging from Chihuahuas with a minimum adult bodyweight of 500 g to breeds weighing over 100 kg, such as Spanish Mastiffs and Saint Bernards [1]. Neonatal mortality in domestic dogs is high, and it is therefore of great interest for breeders to find parameters which may permit the identification of

puppies at risk of neonatal mortality. Dog breeders, both hobbyists and professionals, are continuously trying to improve, and are therefore searching for easily applicable methods to improve neonatal survival. Although, for decades, responsible breeders have been using weight to estimate the wellbeing of puppies and mothers, and experienced breeders may have an estimation of when a puppy of a respective breed is of low bodyweight, no breed-specific charts exist. Therefore, knowledge of physiological birth bodyweight and the neonatal growth curve of the respective breed would be useful for the breeder and the treating veterinarian. Weight is an easily measurable parameter and, confronted with other parameters which might give information on the wellbeing of a neonate, less error-prone. During the daily process of weighing, breeders may further identify particularities which are not as easily visible when observing the puppies in the group. Moreover, for the individual puppy, early contact with humans may have beneficial future effects. The weight curves and growth rate of dogs have been investigated in several studies [2–4]. Weight has an impact not only on health [5] but also on certain behavioral aspects, with larger female puppies being more active and explorative than their smaller counterparts [6]. The growth of puppies after weaning age has been investigated [7,8]. However, little is known about breed differences in birthweight and normal growth patterns within the neonatal phase—the first three weeks of life [9,10]—a very delicate phase of life during which puppies are highly susceptible to health problems. Canine neonatal mortality is estimated to account for 20% of all puppies born until the age of 7–8 weeks, with 75%–90% of deaths occurring within the first three weeks of life [9,11–13]. Correlations between low birth bodyweight (bBW), growth rate within the first 2–4 days of life and neonatal mortality in the canine species have been reported [9,12,14–16] for groups of breeds. Previously published studies on canine bBW used a population comprising puppies of different breeds grouped according to their expected adult bodyweight. To date, there are no rules on how to group dog breeds according to their bodyweight, which leads to important differences in the composition of the populations of these studies. For instance, the Tibetan Terrier has been considered as a dog of small size [16] or of medium size [12], depending on the weight ranges used to describe breeds of either small or medium size. However, no breed-specific bBW cutoff value has yet been established above which a canine neonate is to be considered normal. We included, in our investigated population, puppies of large, medium, and small breeds to permit comparison with previously published results and to show the differences in birthweight and growth. The aims of the present study were to investigate (a) ranges of physiological bBW specific for Bernese Mountain Dogs (BMD), Tibetan Terrier (TT), and Lhasa Apso (LA) breeds; (b) ranges of physiological daily weight gain specific for puppies of the BMD and TT breeds; and (c) possible influencing factors, such as breed, sex, birth sequence and litter size, on birth bodyweight and neonatal growth.

2. Materials and Methods

The breeds included in the present investigation are BMD, TT, and LA. The respective FCI standards describe the BMD as a breed above medium size [17], TT as a breed of medium size [18] and the LA as small-sized breed [19]. A total of 213 puppies from 38 litters of the above three breeds born in two different kennels (BMD and TT in Kennel A and LA in Kennel B) between 1994 and 2018 were included in the statistical analysis (Table 1). Seven puppies of BMD and TT were either stillborn (n = 5) or died during the neonatal period (one due to diffuse hemorrhage on Day 6 and one due to trauma on Day 16) (Table 1), and were excluded from statistical analysis. Information on birth body weight and daily growth within the first 21 days of life was available for a total of 133 puppies of BMD and TT born from Kennel A, while, for 80 LA puppies born within the same period of time from Kennel B, information on birth body weight was available (Table 1). Collected information about bitches included breed, date of birth, parity, and non-pregnant bodyweight. Information available about puppies included breed, date and hour of birth, birth order, sex, and bBW. Birth order describes the position of the puppy in the birth sequence. The day of parturition was considered as Day 0 and the weight on Day 0 was considered the bBW. All puppies of BMD and TT were weighed at least once

daily for the first 21 days of life. In the case of a dystocia, documentation was provided about when during parturition, and which puppies were delivered via an emergency caesarean section.

Table 1. General information on the population.

	Kennel	N Dams	N Litters	N Puppies (Alive/Dead)
BMD	A	3	6	44/5
TT	A	6	16	89/2
LA	B	6	16	80/0

BMD = Bernese Mountain Dog; TT = Tibetan Terrier; LA = Lhasa Apso; N = number.

Information about the health of puppies and the dam was available and taken into consideration to rule out any maternal condition which could endanger neonatal health.

Birth bodyweight of puppies was evaluated in grams (g) and as a percentage of non-pregnant mothers' bodyweight. Values in g are referred to as absolute bBW and values in percentage are referred to as relative bBW. The total litter weight was calculated as the sum of the bBW of puppies of each litter and reported as the percentage of the mothers' bodyweight prior to mating.

The average daily gain of neonatal weight is calculated as the difference between the day of interest and the previous day. The average daily gain is given as the gain in g/day (ADGg) or as percentage of the birth bodyweight (ADG%). Multiplicity describes the time (days) needed for a puppy to double and triple its bBW. Therefore, multiplicity is calculated as the difference between the day of interest and the day of birth.

Dams were weighed prior to mating. Puppies were weighed after colostrum intake on the day of birth. Thereafter, weighing was performed by the breeder at the same time each day. A total of three different kitchen scales were used over the study period. In Kennel A, the scales used were a Tupperware Digital Scale with a range from 1 g to 3 kg (Tupperware, Orlando, FL, USA) and a Soehnle Page Profi digital scale with a range from 1 g to 15 kg. In Kennel B, the scale used was the Zenith Skala analogic scale with a measurable maximum of 26 kg (Zenith, West-Germany, Germany).

Statistical Analysis

Descriptive statistics were performed using Microsoft Excel for Mac 2016, Version 16.16.4. All analyses were performed using SAS (9.4 release, SAS Institute Inc., Cary, NC, USA). Separate analyses by breed were conducted for bBW using a split-plot model with two levels of error. The linear ANOVA model included in the main plot the fixed effects of mother, parity, litter size (three, four, five, six, seven, eight, nine, and more than nine puppies) and litter effect as main error line; in the second plot, the gender of puppies and order in the birth sequence. To allow comparisons among breeds, puppies' bBW and the sum of the bBWs of each litter (expressed as percentage of the bodyweight of their non-pregnant mother) and the average daily gain (ADG%, expressed as percentage of the bBW) were analyzed using the same ANOVA model, which also included the fixed effect of breed. For ADG, separate analyses were conducted for each week of growth. Doubling and tripling of weight were analyzed in the same way. Post hoc multiple comparisons among levels were performed using the Bonferroni correction. A factor was considered to have a significant impact if $p < 0.05$. The impact of the individual mother and the individual litter is referred to as the "mother-effect" and the "litter-effect".

3. Results

The results are presented in three sections. Section 3.1. (general evaluation and bBW) includes a description of the litters, litter size, and the bBW of the three breeds. bBWs are initially given in g and are then presented as percentage of their mothers' bodyweight to allow comparison among breeds. Section 3.2. (average daily gain (g)) features the growth of BMD and TT puppies during the first 21 days of life presented in a descriptive manner. Section 3.3. (average daily gain (%) and multiplicity)

reports the daily gain as percentage of the bBW of BMD and TT puppies to allow comparisons between the two breeds and multiplicity.

3.1. General Evaluations and Birth Bodyweight

The range of litter sizes, mean litter sizes, ranges of mean bBW (g), and ranges of individual bBWs (g) are presented in Table 2. Litter size: the ranges of litter size were 4–11, 3–8, and 3–7 in the BMD, TT, and LA litters, respectively. Neither the age of the dam nor parity had a significant influence on litter size. However, the breed of the dam had a significant influence ($p < 0.05$). Birth bodyweight (in g): the absolute bBW was evaluated separately for each breed and the *p*-values of possible influencing factors are given in Table 3. Puppies of larger litters were born significantly smaller than puppies of smaller litters in BMD and TT. The mother-effect was statistically significant in BMD and TT litters, and the litter-effect was statistically significant in TT and LA (Table 3). bBW (in %): comparison of the bBW in terms of percentage of the mothers' bodyweight showed that BMD puppies were born smaller than TT and LA puppies ($p < 0.0001$). Puppies of BMD weighed 1.3% of mothers body weight. The bBW (%) was 2.5% and 2.1% for LA and TT, respectively ($p = 0.5$). Sex had no impact on bBW (%). Total litter weight: the sum of the bBWs of all puppies of each litter expressed as percentage of their mothers' bodyweight were compared among breeds. The average weight of a BMD litter was 9.6% of their mothers' bodyweight and, therefore, significantly lighter than the weight of LA ($p = 0.0022$) and TT ($p = 0.0008$) litters, which weighed 11.6% and 11.9% of their mothers' bodyweight, respectively. The size of the litter ($p < 0.0001$) and the breed ($p = 0.001$) had a significant impact on the total litter weight.

Table 2. General information on litters and birth bodyweight (bBW).

	Litter Size (Range)	Litter Size (Mean ± SD)	Range Mean bBW ± SD (g)	Range Individual bBW (g)
BMD [a]	4–11	7.3 ± 2.5	462 (±62)–662 (±26)	380–720
TT [a]	3–8	5.6 ± 1.4	150 (±9)–334 (±14)	100–356
LA [b]	3–7	5.0 ± 1.2	137 (±8)–230 (±7)	100–260

BMD = Bernese Mountain Dog; TT = Tibetan Terrier; LA = Lhasa Apso; N = number; range mean bBW (g) = range of mean bBW of all litters; range individual bBW (g) = range of bBW recorded for individual puppies of all litters of each breed; breeds with the same superscript gave birth in the same kennel.

Table 3. Significance of factors influencing the birth bodyweight.

	Litter Size	Mother-Effect	Litter-Effect	Sex of Puppy	Birth Order
BMD	$p < 0.0001$	$p = 0.012$	n.s.	n.s.	n.s.
TT	$p = 0.0003$	$p = 0.0195$	$p = 0.0019$	n.s.	n.s.
LA	n.s.	n.s.	$p < 0.0001$	n.s.	n.s.

BMD = Bernese Mountain Dog; TT = Tibetan Terrier; LA = Lhasa Apso; n.s. = not significant.

3.2. Average Daily Gain (g)

No weight loss within the first 48 h was observed in either breed. The ADGg for the BMD puppies ranged from 60.8 ± 1.4 g/day to 89.6 ± 2.4 g/day and was significantly influenced by litter-effect ($p < 0.0001$) and sex ($p = 0.0202$). When evaluating the influence of sex, the results show that female puppies gained significantly more weight (g/day) than males ($p = 0.0202$). The ADGg of the TT puppies ranged from 29.9 ± 1.2 g/day to a maximum of 47.0 ±1.50 g/day and was significantly influenced by sex ($p = 0.0112$) and bBW ($p < 0.0001$). In contrast to BMD, TT male puppies gained more weight than females. Parity and birth order had no influence on average daily gain in both BMD and TT puppies.

3.3. Average Daily Gain (%) and Multiplicity

Puppies of BMD gained significantly less during the first and second week than puppies of TT. This changed in the third week, yet, with the increase in the ADG% of BMD, the difference lost statistical significance (Tables 4 and 5). Breed and litter size had a significant impact; however, significance was not continuously expressed on each single day of the first and second week. Litter size had a significant impact on ADG% during the first and second week, with puppies of large litters gaining less than puppies of smaller litters (Table 5). Sex had no significant impact on ADG% within a breed, yet, when comparing BMD males with TT males, a significant difference in daily gain could be observed. TT males gained significantly more than BMD males in the first and second week (Table 4). Changes in ADG% are illustrated in Figure 1. When evaluating multiplicity, breed showed a continuous, significant impact from Day 7 onwards ($p < 0.05$). BMD puppies doubled their bBW in 10 ± 0.3 days, which is significantly later than puppies of TT (8.7 ± 0.2 days) ($p = 0.0004$). Litter size had no impact on the multiplicity of bBW, whereas the sex of the puppy did: BMD males needed significantly more time (10.6 ± 0.4 days) to double their weight than BMD females ($p = 0.0486$), TT males ($p = 0.0004$), and TT females ($p = 0.0023$); in every comparison, TT males were the fastest (8.5 ± 0.2 days) in doubling their weight. BMD puppies were slower in tripling their bBW than TT puppies (17.9 ± 0.4 days vs. 14.3 ± 0.3; $p < 0.0001$). The impact of sex between breeds was also statistically significant with regard to tripling. BMD males needed significantly more time (18.6 ± 0.6 days) to triple their weight than TT males and females ($p < 0.0001$) (Table 6).

Table 4. Average daily gain in percentage of the bBW (ADG%).

	N Puppies	1st Week	2nd Week	3rd Week
BMD	44	12.1 ± 0.6 ^{A}	12.99 ± 1.0 ^{B}	17.99 ± 1.5
TT	89	14.66 ± 0.4 ^{A}	16.7 ± 0.7 ^{B}	14.31 ± 1.0

BMD = Bernese Mountain Dog; TT = Tibetan Terrier; N = number; values with the same superscript are significantly different ($p < 0.05$), ^{A}: $p = 0.0005$; ^{B}: $p = 0.0064$.

Table 5. *p*-values of factors influencing the ADG %.

	Breed	Litter Size	Sex	Breed Males (13 BMD vs. 43 TT)	Breed Females (31 BMD vs. 46 TT)
1st week	0.0005	0.001	n.s.	0.0033	n.s.
2nd week	0.0064	<0.0001	n.s.	0.0159	n.s.
3rd week	n.s.	n.s.	n.s.	n.s.	n.s.

BMD = Bernese Mountain Dog; TT = Tibetan Terrier; breed males = BMD males vs. TT males; breed females = BMD females vs. TT females; n.s. = not significant; significant = $p < 0.05$.

Table 6. Multiplicity of birth bodyweight (bBW) and its influencing factors.

	Breed (Mean ± SD)		BMD Males vs. Females (Mean ± SD)		TT Males vs. Females (Mean ± SD)	
Double	BMD: 10 ± 0.3 d TT: 8.7 ± 0.2 d	$p = 0.0004$	Males: 10.6 ± 0.4 d Females: 9.5 ± 0.3 d	$p = 0.0486$	Males: 8.5 ± 0.2 d Females: 8.8 ± 0.2 d	$p = 1$
Triple	BMD: 18 ± 0.4 d TT: 14.3 ± 0.3 d	$p < 0.0001$	Males: 18.6 ± 0.6 d Females: 17.3 ± 0.4 d	$p = 0.1993$	Males: 14 ± 0.3 d Females: 14.7 ± 0.3 d	$p = 0.4356$

BMD = Bernese Mountain Dog; TT = Tibetan Terrier; d = days, significant = $p < 0.05$.

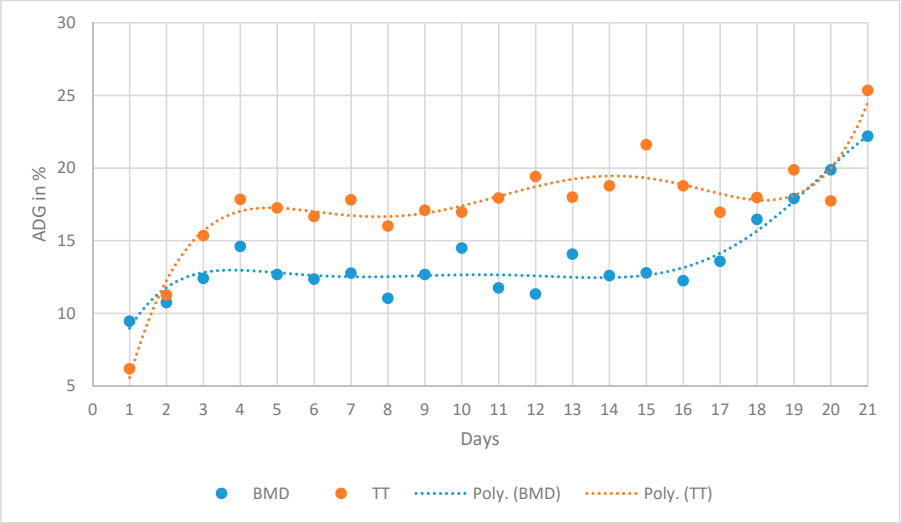

Figure 1. Average daily gain in percent (ADG%) for BMD and TT puppies over the neonatal period; BMD = Bernese Mountain Dog; TT = Tibetan Terrier; poly. = graphical polynomial interpolation of the data.

4. Discussion

4.1. Limitations

The total population size of our study (213) may be perceived as a limitation of the present study when compared to the total number of pups from other studies, such as 2373 [12], 789 [20], or 3292 [21]. However, we focused on only three breeds and, in fact, the number of puppies per breed does not differ considerably. In the present study, 89 puppies of TT, 44 puppies of BMD, and 80 puppies of LA were included. In the abovementioned studies, the number of puppies of BMD was 88 [20] and 196 [21], the number of TT puppies was 2 [21], and the number of LA puppies was 5 [12].

The interval of time between the first and the last litter born in our population may be considered as another limitation, as international breed standards and/or breeding techniques may change with time. Selecting for larger or smaller adult size in certain breeds may have an impact on bBW. Fourteen years is probably a long enough period to obtain significant changes in size and the bodyweight of adult dogs, which may impact bBW. However, the international standards of the three breeds of our study did not change over the last three decades with regard to height and bodyweight. During the 14 years of the study, the three breeders continued to use the same criteria with regard to achieving a certain phenotype by selection [22]. Nutrition of the dam, as well as the housing and handling of both the dam and the puppies, remained unchanged at the kennels during our study. The balances were changed once in Kennel A, whereas in Kennel B the breeder used the same balance throughout the studied period. The influence of the change of balance on the data in Kennel A is difficult to understand in a retrospective manner. We consider the impact as marginal, as the individual birth body weights between litters of similar size weighed with different balances did not differ importantly. Nevertheless, future research and data collection in this field should be obtained using one balance, therefore standardizing data collection to avoid any kind of bias. Over time, slight changes may have been observable in the phenotypical aspects of each of the three investigated breeds. This may be attributed to individual preferences of breeders in the selection of the dam and the sire. Breeders may therefore select animals nearer to the lower or upper range of the described size in the FCI standard of

each respective breed. In this context, we consider the impact of time to be of no importance for the population of the present study as throughout the years breeders maintained the same ideas regarding the phenotype they wanted to achieve by selection. For the future we would consider a breed-specific evaluation of birth body weight and growth as useful, including different breeders for each breed. In this way, any possible influence of personal selection would be decreased to a minimum. We think that complete exclusion of selection-dependent influence on the phenotype of any breed is impossible, as selection and therefore phenotype may differ not only between breeders and changes over time, but also between regions and countries.

4.2. Litter Size

Mean litter sizes are reported to vary widely for the breeds investigated in our study [10,23,24]. The results of the present analysis, as well as the findings of previous reports, are compared in Table 7. The mean litter size of TT in our population is larger than those reported in the literature. Similar results could be found for the BMD breed, as only Groppetti et al. [20] reported a larger litter size. The larger variety in reported litter sizes for BMD may be due to the fact that, in large breeds, the litter size is mainly dependent on the prolificacy of the dam, whereas in smaller breeds, the litter size is further limited by the size of the abdomen. Groppetti et al. [20] described litter size as being influenced by maternal size, weight, age, and parity. In our studied population, age and parity had no significant influence on litter size, whereas breed, and therefore size/weight, had a significant influence ($p < 0.05$).

Table 7. Mean/median litter sizes reported in the current and previous studies.

	Present Study	**Borge et al. 2011 [23]**	**Tønnessen et al. 2012 [10]**	**Leroy et al. 2015 [24]**	**Groppetti et al. 2015 [20]**	**Groppetti et al. 2017 [21]**
BMD	7.3 ± 2.6 [c]	6.4 ± 0.3 [c]	5.7 ± 0.2 [a]	5.5 ± 2.8 [c]	7.8 ± 3.2 [c]	6 [b]
TT	5.6 ±1.4 [c]	5.2 ± 0.3 [c]	5.1 ± 0.3 [a]	n.g.	n.g.	2 [b]
LA	5.0 ± 1.2 [c]	4.9 ± 0.3 [c]	4.7 ± 0.3 [a]	n.g.	n.g.	n.g.

BMD = Bernese Mountain Dog; TT = Tibetan Terrier; LA = Lhasa Apso; n.g. = not given; [a] = litter size at Day 8; [b] = median litter size; [c] = mean litter size.

4.3. Birth Bodyweight

The breed-specific description of bBW for our studied breeds is not uniform, as different authors have reported either the mean, the median, or a range. For BMD puppies, the mean and median bBW has been reported to be 588.9 ± 88.4 g [20] and 541 g, respectively (range 490–600 g) [21]. For puppies of TT, the median bBW has been reported to be 217 g with a range of 215.5–218.5 g [21], whereas for LA puppies, the mean of bBW has been reported to be 242 g with a range of 228–256 g [12]. Our observed means for BMD and LA puppies, and the medians for BMD and TT puppies, are in line with those already reported [12,20,21]. Birth bodyweight has further been evaluated on the basis of different breeds grouped according to their expected adult bodyweight. In these cases, the bBWs of BMD puppies of the present study are similar to the mean and median bBW of puppies of the group of large-sized breeds reported in previous studies [15,16,20]. The importance of breed-specific evaluation becomes evident when comparing our results with the results reported by Gill [12] and Mila et al. [16]. Grouping breeds differently resulted in the TT being either a dog of small size [16] or of a medium size [12]. The bBWs of many of our litters differed from previously reported means and were found to be both above and below [16,20]. The reason for the lack of agreement between Mila [16] and Gill with regard to the lower threshold of bBW may be due to the different compositions of groups of breeds in their studies. Our results are in agreement with those reported by Mila et al. [16], while they are in complete contrast with the findings of Gill [12], who considers puppies' bBW as low if it is lower than one standard deviation below the mean [25]. If we apply this method to our results, eight BMD, 87 TT, and 65 LA puppies should be considered underweight, and therefore at risk of neonatal mortality. In addition, assuming the exclusion of TT and LA puppies of litters which were born with a bBW

of 100–130 g, and therefore on the lower extreme of possible bBW, the remaining 84 and 61 puppies of TT and LA breeds, respectively, would nevertheless be considered underweight, and therefore susceptible to complications and death within the first few days of life [12,16]. When applying the rule using means and standard deviation calculated on the basis of our breed-specific population, only seven BMD, 13 TT, and nine LA puppies weighed less than one standard deviation below the mean. This important difference may show that the application of the method of Grünwald [25] may not be useful if the means and the standard deviation are calculated for breed groups rather than a specific breed, and demonstrates the importance of breed-specific evaluations of this parameter. Therefore, when evaluating the physiological bBW of a specific breed, the calculation of a cutoff value of bBW may be biased if breeds are grouped according to their expected adult bodyweight rather than being evaluated separately. A lack of consistency in possible influencing factors of bBW in the breeds of our study has already been reported [16,20,21,26,27]. We observed a significant effect on bBW of breed (for all three breeds) and of litter size for BMD and TT litters. We did not observe a significant effect of gestational length and maternal factors such as weight, age, size, and breed, unlike what was reported by others [20]. Reports of the significant impact of sex on bBW are inconsistent. A significant sex dimorphism was described by some authors, with males being born significantly heavier than females [21,26,27], whereas other studies found no significant impact of sex on the bBW [16]. Moreover, no significant effect of sex dimorphism on bBW was observed in the present study. The litter-effect is to be considered a complex sum of factors influencing all puppies of the same litter, yet not all litters of the same mother. No clear definition for this effect is currently available, although genetics and environment may well play a role. In our study, the litter-effect had a significant impact on bBW only in TT and LA litters. A significant litter-effect has been reported, yet, due to the study design, it is unclear whether this effect may be present in all breeds [16,27]. Lawler [13] includes the litter-effect when concluding in his study that bBW is influenced by parental age, state of health, placental sufficiency, litter size, gestational nutrition, infections, and environment. Such a conclusion cannot be supported from our results. Further research is needed to clarify the mechanisms behind the litter- or the mother-effect. A negative correlation in comparison of weight ratio between weight of the mother and bBW of the puppies has been previously reported [1] and is supported by the findings of the present study. Puppies of a large breed are therefore born smaller than puppies of a medium or small breed when comparing the bBW in terms of percentage of mothers' body weight. When evaluating the bBW as a percentage of the mothers' bodyweight, the puppies of BMD weighed significantly less than TT and LA puppies. Fiszdon et al. reported that the sum of the puppies' bBWs of a litter should not exceed 15% of the mothers' bodyweight [1]. In the present study, five litters (three TT and two LA) had a weight of over 15% of the mothers' bodyweight, with the heaviest litter weighing 18.4%.

4.4. Neonatal Growth

Comparing our results with the past literature is somewhat difficult, as most studies looking at canine growth rate during the first three weeks of life used a population consisting of different breeds [1,14,15,27]. During the first and second week of life, breed, litter size, and sex, divided by breed, had a significant effect on ADG%. All the factors lose their significance from Day 15 onwards. Mila et al. [15] evaluated growth within the first two days of life (considering it as early growth) and reported a mean growth rate of 3.3% for puppies alive at Day 2, ranging from −4.9% to 13.2%, calculated for a population composed of various breeds of different sizes. The reported early growth rate of puppies still alive at Day 21 (5.1% with a range of −2.2% to 13.2%) [15] is closer to our findings. However, an important difference remains, with puppies of the present population greatly exceeding the ADG% reported by these authors. Mila et al. [15] reported that the early growth rate is predictive of survival within the first 21 days, and proposed a cutoff value of −4%, under which puppies are more likely to die than puppies which lose less weight or gain weight. Such a threshold could not be tested in our study due to the lack of significant neonatal mortality and the difference in the study design.

Our results, which show a small breed gaining more weight each day and growing faster than a large breed, support the findings of Fiszdon et al. [1]. Our results are in agreement with Indrebø et al. [9], who reported an average growth of 8% within the first three days and 12% in the following four days. Furthermore, Hoskins [28] suggested that a daily weight gain of 10% (with an increase of 2–4 g/kg (adult BW)/d during the first five months of life) should be considered desirable. The puppies in the present investigation maintained a weight gain of >10% from Day 2 onwards. Both breeders and veterinarians consider a weight loss of new-born puppies within the first 48 h as normal, yet the reports are inconsistent. Different authors, e.g., Mila and co-authors [14,15] and Bigliardi et al. [26], reported that an initial weight loss during the first 2–4 days of life is not uncommon, and may have a negative impact on neonatal survival. Indrebø et al. [9] considered a weight loss of up to 5% within the first 24 h possible, similarly to Bigliardi and co-authors [26], who described an average weight loss of 11.3 ± 2.3 g (corresponding to 2.3%) in Boxer puppies within the first three days, with larger puppies showing a smaller weight loss. Nevertheless, Bigliardi et al. [26] proposed that the weight loss should in no case exceed 10%, and Münnich [29] advised supplemental feeding if the weight loss in the first 24 h of life is more than 10% of their birthweight. Mila et al. [14] concluded that puppies which lose weight between birth and Day 4 are at a higher risk of death within the first three weeks of life compared to littermates which gain weight during the same period. On the contrary, Hoskins [28] stated that healthy puppies should gain weight continuously. No weight loss could be observed in our studied population. Breeders consider the multiplicity of weight an important indicator of the wellbeing of their puppies and the milk yield and nursing capacity of the mother. Puppies of TT and BMD of the presently investigated population doubled their weight within 8.7 ± 0.2 days and 10.0 ± 0.3 days, respectively. Breed, and sex divided by breed, had a significant impact on the time needed to double weight, with BMD males needing significantly longer than BMD females, TT males and TT females. Overall, TT males were the fastest, with reports of the time needed to achieve doubling of weight ranging from seven to 14 days [1,9,26,30,31]. Considering these reports and the findings of the present study, the perception of breeders that puppies should double their weight around day 10 may be considered realistic.

4.5. General Considerations and Implications for Future Research

Our results, although obtained from a relatively small number of puppies, show the necessity of further research. The evaluation of a large number of puppies and the interpretation of results using breed groups could seem a time- and resource-saving approach to the subject of birth bodyweight and its implications for the breeder and the puppy. Our results, especially considering the application of the rule of Grünwald [25], show how large the difference between a breed-specific and a breed-group based approach is. Experienced breeders may have an estimated cutoff value above which they consider their puppies normal, yet there is no chart which may be used by less experienced breeders or veterinarians who may be confronted with a high number of different breeds. For both inexperienced breeders and veterinarians, such a chart might be useful, as it would permit the appropriate identification of puppies of a low birth bodyweight and increase their probability of survival by receiving special attention, such as additional feeding or complete hand rearing.

5. Conclusions

Comparing the results of different studies on puppies' bBW and their growth rate during the neonatal period is difficult, due to the differences in the composition of the population. There is no generally accepted rule on how to create breed classes on the basis of adult bodyweight. Considering the variety of dog breeds, a breed-specific evaluation of normal bBW and neonatal growth rate is warranted before attempting to create cutoff values below which puppies should be considered at risk. Differences between the results of the present and previous studies show the importance of breed-specific evaluation of these parameters.

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5.2. Appendix 2

animals

MDPI

Review

Prostatic Neoplasia in the Intact and Castrated Dog: How Dangerous is Castration?

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Simple Summary: Castration of dogs is a routinely performed surgery to limit unwanted reproduction and prevent pathologies of the genital tract. Over the last two decades, the number of reports on possible long-term health risks has increased. Pet-owners have easier access to scientific publications and are concerned about reports on increased risks of castrated dogs for neoplastic diseases. Divulgation of results without consideration of study design and inclusion criteria for the studied populations may result in premature conclusions impacting many stakeholders. Our aim is to provide a detailed description of prostatic cancer in the dog and the possible side effects of castration. Age at diagnosis ranges from 8.5 to 11.2 years in both intact and castrated dogs. A cytological or histological exam is needed to confirm a suspect. Most dogs already present metastasis at the time of diagnosis which makes prognosis generally poor, also if lung metastasis reportedly has no negative impact on the survival time. Castrated dogs with prostate cancer have been reported to live longer than intact ones. We conclude that until today, we knew too little to exclude routine castration of adult male dogs under six years of age from the veterinary practice due to concerns of causing prostatic neoplasia.

Abstract: Elective gonadectomy in the dog is a topic of interest for clinicians, pet-owners, and society. Although canine prostatic neoplasia (CPN) has a low incidence (0.35%), reports of an increased risk for castrated dogs attract attention and cause concern in pet-owners. Our aim is to provide professionals and non-professionals with a detailed description of this possible side effect of gonadectomy in the dog. The mean age at diagnosis of CPN ranges from 8.5 to 11.2 years. Medium to large size breeds are more frequently affected. Symptoms and findings of non-invasive examinations are not pathognomonic, therefore, cytological or histological examinations are needed for diagnosis. Overall, the incidence of metastasis reaches up to 80%, yet lung metastasis reportedly has no negative impact on median survival time (MST). It has been reported that castrated males have a significantly higher MST than intact males. Differences in inclusion criteria for studied populations make a comparison of studies difficult. Citation of odds ratios without consideration of the context of the reference may result in premature conclusions. We conclude that elective gonadectomy of adult male dogs under six years of age cannot be excluded from the veterinary practice due to concern of causing CPN until clear and strong evidence is available.

Keywords: dog; castration; gonadectomy; prostatic neoplasms; prostate cancer

1. Introduction

Elective gonadectomy is most commonly used for the prevention of unwanted reproduction in cats and dogs. Especially in countries with a high number of stray animals, neuter-spay-programs have been initiated and owners have been advised to have a surgical castration performed on their pet. Although there are clear and well-documented advantages of gonadectomy in the dog, the reports

of possible disadvantages increased over the last two decades and cause concern in a large group of pet-owners. One such possible reported disadvantage is the canine prostatic neoplasia (CPN). Canine prostatic neoplasia has been attracting the interest of researchers and clinicians due to its similarities with human prostatic neoplasia despite a much lower incidence in dogs (0.35%) compared to humans (30%) based on necropsy studies [1,2]. The role of neuter status has been the object of ongoing debate because of claims that gonadectomy may increase the incidence and/or hasten the progression of prostatic neoplasia in male dogs. Over the last two decades, several studies have shown a statistically significant increased risk for the diagnosis of various neoplastic conditions in castrated males and females compared to intact dogs [3–8]. These findings, among others, have initiated a discussion on the advantages and disadvantages of elective gonadectomy in the dog. However, a definitive explanation of how the lack of gonadal hormones may influence the development of neoplasia in reproductive or non-reproductive tissues has yet to be provided. Several reviews have been published over the years on the advantages and disadvantages of gonadectomy in dogs [9–13]. However, two factors have had an impact on the quality of these publications: editorial limitations of the publication's length and the tendency to report only the odds ratio, without considering the study design and the conclusions drawn by the authors of the cited publication [10,12,14]. Therefore, the role of neuter status on the development of canine prostatic neoplasia has been considered as a risk factor not only for prostatic neoplasia but also for other neoplastic disorders in male dogs, rising concerns on whether or not elective gonadectomy in dogs should be regarded as a safe procedure. The aim of this paper is to provide an in-depth review of current knowledge on canine prostatic neoplasia and the role of the castrated male.

2. Incidence, Prevalence, and Signalment

A summary of the studies on canine prostatic neoplasia (CPN) is provided in Table 1. Weaver et al. [1] reported an incidence of canine prostatic neoplasia of 0.35%, more recently Bryan et al. [15] reported a maximum annual incidence of 0.93%, although the latter combine prostatic tumors with urethral and bladder transitional cell carcinoma (TCC). The incidence of CPN is not reported in any other study on this topic. Furthermore, the total number of animals is not always featured, as well as the type of neoplasia and the castration-diagnosis-interval (CDI). Although the total number of animals within a study varies widely (26–72,300 dogs), the retrospectively calculated prevalence (Ntotal/N of CPN = calculated prevalence) in most papers is <1% of the total population studied (Table 1). In the cases of Aquilina et al. [16], Troisi et al. [17], and Donato et al. [18], the study design may be an explanation for the high calculated prevalence of CPN. The total number of animals reported by these three publications is the number of animals included in the study rather than the total number of animals seen at the hospital during a certain period, making these values hardly comparable to prevalences calculated on the total population of dogs examined or autopsied. Bryan et al. [15] do not provide any information on overall incidence, yet report an annual occurrence of CPN ranging from 0% to 0.93%, with a mean and median annual occurrence of 0.45% and 0.47%, respectively. An important aspect of the signalment of dogs affected by prostatic neoplasia is age. Just as benign prostate hyperplasia (BPH) in intact males and pyometra in intact females, also prostatic neoplasia is a disorder mainly diagnosed in the elderly dog, with an average age of 8.5 to 11.2 years at the time of diagnosis (Table 1). Canine carcinomas are typical of elderly age [19] and it has been hypothesized that physiological changes due to aging may have an impact on the initiation of CPN development [20]. Although the median or mean body weight is rarely mentioned, medium to large size breeds are more frequently affected by CPN than small or miniature breeds [1,21,22]. No breed predilection has ever been confirmed. However, an increased risk/odds ratio of CPN has been reported for Shetland Sheepdogs, Scottish Terriers, Bouvier des Flandres, Dobermann Pinscher, and dogs of mixed breed [6,15,23,24].

Table 1. Overview of the studied populations and the signalment in chronological order of publication.

Studies	N_{total}	N_{PD}	N_{CPN}	Age (y)	BW (kg)	Prevalence	I/C_t/C/U	CDI (Years)
[25]	>500		3			0.6		
[19]	7248		8			0.11		
[26]			6					
[27]			20	10.1 [A]			16/3/1/0	
[28]		140	22	9.3 [A]			0/0/1/21	
[1]	~4500	430	15	9 [A]	27.8 [A]		15/0/0/0	
[23]	1483/13,633		14	8.5 [A]		0.94/0.05	7/0/7/0	0.75-5 [E]
[29]			43	9.8 [A]			14/10/19/0	GI: 5.5 [C]; 7 [D] GII: 9.6 [C];6.5 [D]
[30]			31	10 [B]			21/0/10/0	6.4 [D]
[24]	7069	177	13	10 [A]		0.18	5/1/7/0	2–8 [E]
[31]			15				4/0/11/0	
[16]	199		25	9.4 [A]		12.6	4/0/11/10	
[32]			76	10 [B]	20.5 [B]		28/12/36/0	7 [D]
[33]			19				9/0/10/0	
[6]	15,363	431	56	9.9 [A]		0.3	30/0/26/0	1–10 [E]
[34]			70	10 [B]			21/0/49/0	
[35]			17				5/0/12/0	
[36]	8179		2			0.02		
[37]		25	3				1/0/2/0	
[15]			1384					
[38]		111	50					
[17]	26	18	5			19.23		
[39]	72,300	418	11	11.2 [A]		0.015	7/0/4/0	
[18]	61	51	29			47.5		
[40]			10	9.3 [B]	25 [B]		0/0/10/0	
[22]			67	9.5 [B]	23.3 [B]		7/0/60/0	

N total = total number of animals in the population; NPD: number of animals with prostatic disorder; NCPN: number of animals with prostatic neoplasia; y = years; BW = body weight; A = mean; B = median; P = prevalence calculated based on total number of animals divided by number of dogs with CPN; I = intact; CT = castrated as treatment; C = castrated; U = unknown; CDI = castration-diagnosis-interval; GI = Group I (castrated >12 months of age and >3 years prior to diagnosis); GII = Group II (castrated <12 months of age and >3 years prior to diagnosis); C = mean CDI; D = median CDI; E =range of CDI.

Role of Neuter Status

Obradovich et al. [29] and Bell et al. [30] were the first to investigate the role of castration in the development of CPN. A causal relationship was not postulated and until the end of the century, castration was widely believed to be the most appropriate treatment for any prostatic condition. However, over the last 20 years, there has been increased attention to the health consequences of gonadectomy in small animals and also CPN has been regarded as being influenced by castration in many reviews [9,10,12,14,41,42]. The sample size, number of dogs with CPN and CDI varies considerably among retrospective studies (Table 1). Unfortunately, few authors provide detailed information on (a) whether or not dogs were castrated, (b) when castration was performed during life, (c) the size of the reference population, (d) how long before the diagnosis the dog was castrated in order to be included in the castrated group. Sadly, most studies fail to provide information on (b) and (d). With regard to (d), only two authors provide detailed and complete information: Teske et al. [6] considered dogs as castrated if they were gonadectomized at least 100 days prior to diagnosis, yet reports a CDI ranging from 1–10 years; Obradovich et al. [29] instead used three years as a cut-off value after which the authors considered dogs as castrated at the time of diagnosis. If dogs were

castrated less than three years prior to diagnosis, they were considered as sexually intact at the onset of prostatic disease. The majority of authors include castrated dogs in the group of castrates in their statistical evaluation despite lack of information on how recently (prior to diagnosis) castration was performed [16,23,27,33,34]. Sorenmo et al. [34] presented one of the few publications in which the number of castrated animals was higher than the number of intact animals. They report a median age at castration of two years (ranging from two to 14 years); information was unavailable in five cases, as was a median or mean value of the CDI. The interval between castration and diagnosis of prostatic neoplasia is vital information to investigate a possible correlation between gonadectomy and the incidence of prostatic neoplasia. Furthermore, the criteria on when a dog is included in the group of castrates should always be clearly stated. Age at diagnosis did not differ between castrated and intact dogs in the publications of Teske et al. [6] and Bell et al. [30], yet Bryan et al. [15] report that castrated dogs with prostatic neoplasia were significantly older at the time of diagnosis. Perhaps the decrease of prostatic size after castration that subsequently delayed onset of prostatomegaly related symptoms may cause a delayed diagnosis. Cornell et al. [32] instead report no difference in age at diagnosis.

3. Clinical Signs

Clinical signs may be grouped into gastrointestinal (GI, straining to defecate up to complete constipation, tenesmus, and deformed feces), urinary (straining to urinate, hematuria, incontinence, polyuria/polydipsia (PU/PD) and dysuria), and locomotor (hindlimb weakness, lameness and pain) [1,24,30,32,38,39]. Affected dogs may also show generalized symptoms such as anorexia, emaciation, weight loss, and abdominal pain. Whereas GI- and urinary tract signs may be easily explained on the basis of prostatic topographic anatomy, skeletal problems, and general signs are often secondary to metastatic disease. Table 2 shows the percentages of dogs that have at least one of the above-mentioned clinical signs. The majority of studies providing information on clinical signs describe almost all locomotor but not all systemic signs as being linked to the presence of metastasis. Bell et al. [30] report that urinary tract signs without GI-signs were found in 40% of neutered dogs but only in 9.5% of intact males, whereas GI-signs without urinary signs were found exclusively in intact patients, accounting for 33%, although the differences were not statistically significant. Dogs affected by BPH have been reported to show hematuria and urethral discharge, yet only a few of them show stranguria or dysuria [24] Although a high percentage of urinary tract signs is reported in dogs affected by prostatic carcinoma, the same clinical signs may be found in dogs affected by other prostatic disorders and may, therefore, not be considered CPN specific [24,41]. However small, animal practitioners should always consider CPN as a differential diagnosis in male dogs with severe urinary or GI-signs.

Table 2. Percentage of clinical signs in dogs affected by prostatic neoplasia in the respective studied population.

Studies	GI (%)	Urinary Tract (%)	Locomotor (%)	Systemic (%)
[27]	45	65	55	70
[1]	60	73	27	33
[30]	45			35
[24]	31	61	7.7	23
[32]	30	62	36	42
[38]	22	30	16	16
[43]	7.5	50	12.5	5
[39]	82			46
[40]	24	96		32

GI = gastro-intestinal.

4. Diagnosis

Following a thorough clinical history and clinical exam, the performance of digital palpation of the prostate per rectum may give indications on the presence of a prostatic disorder [1,23,27]. In healthy dogs, digital rectal palpation should not elicit pain [44]. A prostate gland affected by neoplasia may be felt as an irregular, immobile, asymmetrical mass that is often (but not necessarily) painful [21]. Historically, radiology has been used to assess prostatic size [1,23,27,28] which is normal when its diameter does not exceed 50% or 70% of the width of the pelvic inlet [45,46]. Prostatic enlargement, calcification of the gland as well as bone and lung-metastases may be found in a latero-lateral projection [47]. Ultrasound and radiographic examination in dogs affected by CPN may show an enlarged gland with focal to diffuse hyperechoic areas, foci of mineralization, loss of physiologic prostatic contour, and locoregional lymphadenopathy [30,48,49]. A cytologic or histologic examination is necessary to confirm a suspected diagnosis of CPN. Cytological samples may be collected either by transabdominal fine-needle aspiration biopsy (FNAB) under ultrasound-guidance [50], or by cell collection via a urinary catheter following a prostatic massage. The collection of specimens for histology may be performed during laparotomy either as a punch-biopsy or excisional biopsy. Reliability of FNAB has been reported to vary between 50% [51] and 80% [37,52]. A biopsy followed by a histological examination resulted in correct diagnosis in 66–80% of cases [37,49,52,53]. The reliability of transabdominal FNAB and cell collection via a urinary catheter has been evaluated with the results being compared to biopsy results. FNAB correctly identified 63% of histologically diagnosed neoplasia, whereas catheter aspiration diagnosed 55% of cases correctly [53]. Transabdominal FNAB is currently not recommended by some authors, due to the possibility of dissemination of TCC tumor cells along the needle pathway [52]. Although excisional biopsies are contraindicated in the presence of acute prostatitis and abscesses [54], the technique is still considered the most reliable to diagnose CPN. Histologically CPN may be divided into prostatic adenocarcinoma (PACA), prostatic carcinoma (PCA) and tumors of mixed morphology [27]. PACA has been described as being either Type A (adenocarcinoma) or Type B (undifferentiated adenocarcinoma). Type A is subdivided into intra-alveolar proliferative or small acinar and Type B into syncytial or discrete epithelial [27]. The intra-alveolar proliferative type is reported to be the most common histological subtype. Tumors of mixed morphology were described as more frequently associated with metastasis [38]. Table 3 illustrates the prevalence of the different types of prostatic neoplasia.

Table 3. Frequency of different histological types of canine prostatic neoplasia in the respective studied population.

Studies	Adenocarcinoma (%)	Undifferentiated Adenocarcinoma (%)	Carcinoma (%)	Mixed-Morphology (%)	Other (%)
[27]	80	20			
[32]	36			53	11
[15]	43		29		28
[55]			25	75	
[38]			62	38	
[22]	54				

A marker expressed by the basal cell layer of the human prostate, P63, was found also in cases of CPN and the positivity of CPN for p63 was associated with a significant shortening of survival time [56]. Canine prostatic neoplasia with p63-positivity is suspected to present a distinct entity rather than a subtype of canine prostatic carcinoma [56]. Due to its possible implications on metastatic behavior, we consider the histological evaluation of CPN of utmost importance and, therefore, the biopsy is preferable towards cytological evaluations. The availability of an early marker for CPN would have an important impact on treatment and, therefore, the outcome and prognosis of the disease. Unfortunately, no early diagnostic marker for CPN is currently available. Canine prostatic arginine esterase (CPSE) is marketed as a diagnostic marker for prostatic disorders, yet until today, its measurement does not permit a distinction of CPN and other prostatic pathologies [31,57,58].

Role of Neuter Status

Different authors describe the castrated male as being more frequently affected by poorly differentiated prostatic neoplasia [30,32,48,55]. Although Bryan et al. [15] report an increased overall risk for CPN in castrated dogs, PACA was the subtype with the least risk.

5. Metastatic Behavior

Prostatic neoplasia in both men and dogs is often associated with bone metastasis, giving the tumor the reputation to metastasize fast and frequently to the skeletal system [20]. However, when considering the reports on metastasis in dogs affected by CPN, lungs and regional (iliac) lymph nodes are affected more frequently than bones [1,22,27,30,32,34,43] (see Table 4).

Table 4. Frequency and site of metastasis in dogs affected by prostatic neoplasia in the respective studied population.

Studies	Iliac Ln (%)	Lung (%)	Bone (%)	Urinary Bladder (%)	Other Organs (%)	Total Frequency
[27]	75	65	35	50	5–35	15/20 dogs
[1]	87	33	20	60		13/15 dogs
[30]	33	62	15	18	11–33	13/25 dogs
[20]			24			
[32]	51	50	22		1–9	61/78 dogs
[34]	43	32	25.5			45/70 dogs
[38]	33.3	41.7			8.3–25	12/50 dogs
[43]	43	43	14			14/28 dogs
[22]	28	15	2		1	26/67 dogs

Ln = lymph node.

The overall incidence of metastasis in dogs with CPN ranges from 16% [18] to 80% [32]. Canine prostatic neoplasia of mixed morphology was associated with an increased metastatic frequency [32,38]. These authors hypothesize that also the age of the patient influences the biological behavior of CPN. Prostatic tumors in the dog which stained positively for CK7 were reported to have both a higher overall metastatic frequency ($p = 0.04$) and a higher frequency of bone metastasis ($p = 0.03$), compared to CK7-negative CPN [34]. Surprisingly, lung metastasis has been reported to have no negative impact on median survival time (MST) [22]. Further research is needed to reevaluate the importance of the presence or absence of metastatic disease on treatment-choices and prognosis.

Role of Neuter Status

Castrated dogs had a statistically significant increase in lung metastasis frequency [30], yet Cornell et al. [32] report no difference in metastatic frequency or prevalence of skeletal metastasis between intact and castrated dogs. In the study of Sorenmo et al. [34] dogs with CK7 positive prostatic neoplasia had been castrated at a younger age than dogs with CK7 negative prostatic neoplasia; one could speculate that castration at a younger age increases the risk of metastatic disease and bone metastasis in cases of CPN.

6. Treatment and Outcome

Despite the improvement of prognosis in men thanks to recent advances in the diagnosis and treatment of prostatic neoplasia, prognosis, and outcome of this condition in dogs remain poor [42]. Early studies report a survival time in affected dogs of only a few days [1,20,23,30]. Treatment options include surgical removal of the tumor by subtotal prostatectomy or complete removal of the gland by total prostatectomy [40,59]. Radio-or chemotherapy protocols are described also for dogs affected by prostatic neoplasia [49]. However, because of diffuse metastatic disease at the time of diagnosis, the above treatment protocols are rarely applied [30,32,48]. Recently, Ravicini et al. [22] treated 67 dogs with various degrees of prostatic neoplasia either with nonsteroidal anti-inflammatory drugs (NSAIDs) (46%), with chemotherapy (6%), or with a combination of both (48%) (Table 5). None of the dogs in

this study received surgical treatment or radiotherapy, yet 33% of these dogs showed improvement in clinical signs. The median survival time (MST) was 82 days with a range of 9–752 days. None of the 18% of dogs showing prolonged survival (>7 months) had metastatic disease. Lung metastasis had no negative impact on prognosis and MST, yet MST was negatively influenced by the sexual status with intact males living for a significantly shorter time than neutered males. Considering that already Gupta et al. [60] and Tremblay et al. [61] proposed a potential association between the presence of cyclooxygenase (COX)-2 and prostate carcinogenesis, the results of Doré et al. [62] on the enzyme expression and estrogen in the canine prostate may be considered of great importance due to its potential impact on the treatment of prostatic neoplasia. Surgical treatment is possible although urinary incontinence is reported as an important post-operative risk, mainly following total prostatectomy [21]. Bennett et al. [40] described permanent incontinence in eight out of 23 dogs treated with total prostatectomy. Only two of these incontinent dogs were affected by a prostatic carcinoma, whereas the other six dogs were diagnosed with a transitional cell tumor. L'Eplattenier et al. [59] used a surgical laser to perform a subcapsular partial prostatectomy (Table 5). Due to the incomplete removal of neoplastic tissue, all dogs in this study received a post-operative treatment with Interleukin-2 (which, however, was considered palliative rather than curative); incontinence was absent in all cases and the median survival time was 103 days with a range of 5–239 days [59]. In this paper [59] none of the animals had metastatic disease at the time of treatment. Bennett et al. [40] treated 25 dogs with total prostatectomy (Table 5). Of these 25 dogs, 15 were diagnosed with a TCC, nine with adenocarcinoma and one dog with undifferentiated carcinoma. The overall MST was 231 days, ranging from 24 to 1255 days, with one- and two-year survival rates equal to 32% and 12%, respectively, without significant difference between dogs affected by TCC and dogs affected by adenocarcinoma. Only one dog in this study had confirmed metastatic disease at the time of diagnosis. In order to improve the prognostic accuracy of CPN, the Gleason [63] scoring system was tested on its viability in the canine species [43]. A scoring system of 1–5 is given to the primary (most prevalent) and the secondary (second most prevalent) pattern found in a histological specimen. Tissue with grades 1–3 resemble normal prostatic tissue, while grades 4 and 5 refer to tissues with abnormal glandular architecture. Similar to men, dogs with metastatic disease had a Gleason score of 10, and therefore, a worse prognosis [43]. The Gleason scoring system may be used also in the canine species [43].

Table 5. Illustration of possible treatments of CPN.

Studies	Primary Treatment	Adjuvant Treatment	N Treated Animals	MST
[59]	Partial prostatectomy	Local interleukin-2 and systemic meloxicam	8	5–239 d (103 d)
[22]	NSAIDs; NSAIDs and chemotherapy; Chemotherapy	Tramadol, maropitant, lactulose, amantadine, mirtazapine, gabapentin	67	9–752 d (82 d)
[40]	Total prostatectomy	NSAIDs, opioids, tramadol, ketamin or acetaminophen	25 (15 TCC, 9 carcinoma, 1 cystadenocarcinoma)	TCC: 34–664 d (189 d); Adenocarcinoma: 24–1255 d (248 d)

NSAIDs = nonsteroidal anti-inflammatory drugs; N = number; TCC = transitional cell carcinoma; MST = median survival time; d = days.

Role of Neuter Status

Ravicini et al. [22] report that intact males with prostatic neoplasia had a significantly shorter median survival time than castrated males with prostatic neoplasia. Interestingly, this finding does not support the theory that prostatic neoplasia in castrated dogs is of a more aggressive nature or that castration favors tumor progression [6,30,32].

7. Prostate Cancer in the Human

Prostate cancer in the human (HPC) and its counterpart in the dog, although similar in many aspects, show important differences that may explain the improvement in outcome in the human and the continuing difficulty to obtain improvement in veterinary medicine [27,64]. In 1995, prostatic cancer in the human man was reported to be the most frequently non-cutaneous tumor diagnosed in the United States of America, accounting for >400,000 deaths each year [65], whereas in 2005, a rate of diagnosis of 232,090 and a number of 30,350 deaths was reported [15]. Worldwide the estimate of new diagnosis of prostatic cancer in the human accounts for 903,500 males, with a difference between more and less developed countries [66]. This difference, as well as the improvement in mortality rate, may be attributed to differences in the availability of diagnostics and treatment services [66]. The rapid rise of the number of diagnosis in the 1990s, as well as the combination of a high number of diagnosis and a low number of mortality in 2008, may be mainly attributed to the increased availability of screening tests such as the prostate-specific antigen (PSA), which permit early detection of prostatic cancer in the human [66]. One of the similarities between human and canine prostatic cancer is the age of diagnosis. The use of an algorithm that changes the chronological age of dogs into physiological age permitted the comparison between age at diagnosis of humans and dogs [67]. The mean and median of physiological age at diagnosis for CPN was 67 years and 73 years, respectively, whereas humans were diagnosed with a mean age of 70 years [67]. Further findings of high-grade prostatic neoplasia in the dog as well as the tendency to develop bone metastasis gave reason to believe that the dog may be a valuable animal model for human prostate cancer [16], especially for the late, androgen-independent stage with metastasis in lymph nodes, lung, and bone [68]. Although these similarities are important, one of the main differences between CPN and HPC is the initial hormone responsiveness of HPC [64,69]. This sensibility on testicular hormones gives the possibility of hormone ablation therapy. Instead, CPN was reported to be not influenced by hormone ablation and the absence of influence of testicular hormones may be a possible explanation for the lack of protective effect of castration towards its development [29]. Further, the high number of post-mortem diagnosis of HPC (in >40% of necropsied men) may be considered an indication for a high number of latent or slow-growing prostatic tumors that have not been reported for CPN [6,27].

8. Discussion

8.1. Etiopathology

The etiopathology of the canine prostatic neoplasia remains until the present day unclear. Immunohistochemical methods were used to either distinguish certain types of CPN [35] or to provide insight on possible initiating or modulating factors [53,62,70]. The work presented by Shidaifat and co-authors [70] on the expression of vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) in prostates of castrated animals are in contrast with the assumption of different authors, that castration of the male dog may have an impact on the development of prostatic neoplasia. A decade after this conclusion was made, Fonseca et al. [56] hypothesized that p63-positive tumors may be a distinct entity rather than a subtype of prostatic carcinoma. This finding may be used as motivation to investigate in the future, if the tumors of castrated males are more likely to be p63-positive and may, therefore, follow a different etiopathological pathway than the neoplasia of intact males.

8.2. Morphology of Canine Prostatic Neoplasia

Studies published in the early 21st century started to provide information on morphological subtypes of CPN. A confounding factor in the denomination of prostatic neoplasia is the fact that studies, such as Bryan et al. [15] included the transitional cell carcinoma (TCC) in the group of prostatic neoplasia. Studies that do not provide information on the morphological subtypes of CPN also did not state whether they included or excluded the TCC of the bladder and/or the prostatic urethra [1,6,24,59].

Inclusion of TCC into the group of CPN may result in an incorrectly calculated or reported incidence of prostatic neoplasia in the dog. MacLachan et al. [71] report PACA and TCC as the main neoplasia found in the canine prostate. Due to its different cellular origin, TCC should be considered a neoplasia of non-prostatic origin which has to be excluded from statistical evaluations of incidence, outcome and MST of prostatic neoplasia in the dog. Knowledge about the morphological subtypes is of importance due to the suspected difference in biological behavior, such as metastatic behavior [32,38], and the difference in frequency in the groups of intact and castrated male dogs [30,32,48,55]. Leav and Ling [27] provided very early in prostatic research, a classification of the morphological subtypes, which remains valid until the present day. The most common morphological subtype in both intact and castrated animals is the intra-alveolar proliferative adenocarcinoma (Type A1) [33]. Gobello et al. [48] instead report that castrated males are more often affected by poorly differentiated prostatic neoplasia. Cornell et al. [32] and Palmieri et al. [38] report a trend towards an increased overall metastatic frequency of tumors of mixed morphology. It is due to these results that we consider the information on the morphological subtype vital for further research.

8.3. Signalment and Clinical Signs

For both owners and veterinarians, it is important to know the groups at risk for CPN development and their clinical presentation. First of all, age seems a factor of prime importance in the development and diagnosis of prostatic neoplasia in the dog, just as it is in men. The mean age at diagnosis ranges in the various studies between 8.5 years and 11.2 years [1,6,16,23,24,27–29,39] (Table 1). In most studies, dogs affected by CPN are reported to be either six years of age or older [1,6,15,23,24,27,30,32]. Polisca et al. [39] suggest further to start with a geriatric screening of the prostate in male dogs from 6–7 years on. Additionally, BPH is a prostatic disorder mainly found in elderly dogs and is found with increasing frequency with increasing age. Therefore, a correlation between the changes during aging and the development of both BPH and prostatic neoplasia may be hypothesized [48]. BPH and prostatic neoplasia share therefore similarities not only in the age distribution but may be present concomitantly in intact male dogs [49]. Nevertheless, the presence of BPH is not a predisposing factor for the development of prostatic neoplasia [42]. Although few studies provide information on the mean or median body weight of dogs diagnosed with prostatic neoplasia, the majority of researchers report a population of mainly medium- to large size dogs [1,21,22,32,40]. Breed predispositions are widely negated, yet dogs of Shetland Sheepdog, Scottish Terrier, Bouvier des Flandres, Dobermann Pinscher, and mixed breeds are mentioned more frequently than other breeds [6,15,23,24]. Due to the overrepresentation of medium to large size dogs in many studies, we suggest consideration of dogs of medium to large size over six years of age as at increased risk for CPN regardless of their sexual status. In dogs, clinical signs caused by prostatic neoplasia have been reported as well for other prostatic disorders, and may therefore not be considered pathognomonic [24,41]. However, both in CPN as well as in other prostatic disorders, clinical signs may easily be explained by the topographical location of the gland. Neoplasia of the prostatic gland has been reported to be locally invasive and capable of compromising surrounding organs with increasing size [24,28,29]. Urinary tract signs, although possibly present in other prostatic disorders, are often reported as the prime clinical signs in dogs affected by CPN [1,23,24,32,38]. Locomotor and systemic signs in dogs with CPN may be considered in a high percentage of cases as caused by metastatic disease or progressed local infiltration of the prostatic neoplasia [32,41]. Alterations found during digital rectal palpation may indicate the presence of a prostatic disorder, yet none of these alterations may be considered pathognomonic for CPN [1]. An easily palpable prostatic gland in a dog that was castrated early in his life may already be considered a pathological finding [49]. Although digital rectal palpation is an inexpensive clinical tool, it may provide information indicating the prostate gland as a source of the clinical signs. We, therefore, advise performing digital rectal palpation during every general examination of a male dog, regardless of its sexual status. Ultrasound examinations and radiographic studies are useful tools to evaluate the structure, size and presence or absence of prostatic or paraprostatic cysts [23,30,41]. Calcification

of the prostate is frequently found in diagnostic imaging of CPN [30,41]. Although the finding of calcifications is supportive of a suspected diagnosis of prostatic neoplasia, they may be present also in other prostatic disorders [72]. The only reliable tool to confirm the suspected diagnosis of CPN remains the histological or cytological evaluation of prostatic specimens obtained by various methods [1,27]. We regard fine-needle aspiration biopsies as not advisable in routine examinations of dogs at risk of CPN, yet necessary in cases of a substantial suspect of the presence of CPN. Therefore, we advise routine general clinical examination of at-risk dogs in the absence of clinical signs, including rectal palpation and diagnostic imaging [39].

8.4. Diagnosis

Although advances in the understanding of prostatic neoplasia have been made, the search for a diagnostic marker for CPN, similar to the PSA serum concentration in men, continues [23,30,57]. The availability of such an early marker may improve overall outcome, considering that today 60–80% of dogs are already affected by the gross metastatic disease at the time of diagnosis [29,32]. Until an early marker is discovered and validated, we consider screening ultrasound examinations in at-risk dogs recommendable and in case of suspected CPN, a cytological or histopathological examination should be performed. To the present day, cytological and histopathological examinations of prostatic specimens remain the only methods to confirm a suspected CPN [1,27,41]. In human medicine, the Gleason score is a widely used and recognized method to predict the prognosis of prostatic cancer [16,73,74]. Palmieri et al. [43] applied this method in cases of CPN, suggesting that the Gleason score and grading system may be useful in prognostic prediction also in the dog. Nevertheless, the presence or absence of metastasis remains a very important factor in prognostic evaluations and ultimately influences also the choice of treatment [22,40,43]. In the dog, the organs most likely affected by a metastatic disease are the regional lymph nodes and the lung [1,22,27,30,32,34,43]. A radiographic study or if possible, a computed tomography (CT) scan, should be included in the staging of CPN, and may potentially be performed also before cytological or histological results are available [22,32,40]. Considering the frequency of urinary tract signs, we consider it useful to include a urin analysis in the clinical protocol to rule out concomitant urinary tract infections or other problems related to the urinary bladder or the lower urinary tract [30]. Considering the improvement in mortality rate in human prostate cancer following the initiation of screening examinations using an early marker, we consider the search for an early marker of utmost importance towards the improvement of prognosis in CPN.

8.5. Treatment and Prognosis

Treatment of prostatic neoplasia consisted initially in castration, estrogen therapy and therapy with antibiotics and NSAIDs [22,30,41]. Although castration may have a beneficial effect on clinical signs due to the reduction in glands size, ultimately it does not impact the survival time of the animal. The reduction of prostatic size and, subsequently, the improvement of signs may be mainly due to the concomitant presence of BPH in intact dogs with prostatic neoplasia [49]. Total prostatectomy and subtotal prostatectomy showed a beneficial impact on the survival time of the animals, yet mainly, if not only, in cases without metastasis [40,59]. Chemotherapy became more frequently used in veterinary medicine over the last decades for various neoplastic disorders. However, the intensity of the treatment plan, its economic cost and the not yet widely acknowledged usefulness in terms of curative outcome, make it a rare treatment choice [10,22]. Ravicini et al. [22] used chemotherapy in combination with NSAIDs and supportive therapy in their CPN cases. Just as in Bennett et al. [40] and L'Eplattenier et al. [59], the most important factor of impact on the MST was the presence or absence of metastasis at the time of diagnosis. Although the MST was prolonged up to over seven months in dogs without metastatic disorder at the time of diagnosis, the patients ultimately died or were euthanized due to prostatic neoplasia and its complications. The high percentage of metastatic disease at the time of diagnosis highly influences the decision of the owner to opt for euthanasia at the time of diagnosis or shortly after. In the study of Obradovich et al. [29], 81% of the owners refused treatment. Clinical

signs such as pain and discomfort obviously should be treated and euthanasia should be performed if the dog's suffering exceeds his quality of life, yet to the present day, no data is available on how long dogs may survive if adjuvant therapy is the only treatment they receive.

8.6. Castration

Castration is a routinely performed surgery in the dog, mainly but not exclusively to avoid unwanted offspring. Removal of the testicles subsequently results in the removal of testosterone and its active metabolite DHT from the general circulation. The removal of these hormones results in a decrease in the size of the prostatic gland and a decrease in sexually motivated behavior and infertility [41,75]. Hormonal implants have a similar yet reversible effect on the canine organism [76], yet until today, no studies have been designed to evaluate a possible increased risk for tumor development in implanted, and therefore chemically and reversibly castrated animals. Considering that more than 95% of dogs over the age of nine years are or will be affected by BPH, castration is also used for treatment and/or prevention of this disorder [48]. Surgical castration causes a 70% reduction in the dimension of the gland. Although this process begins already 7–14 days after castration, complete involution of the gland may be expected by four months [41,77]. Dubé et al. [78] report that, although the reduction in size in dogs affected by CPN was less than in unaffected dogs, it was nevertheless present. This decrease in size may be attributed mainly to the reduction of epithelial cells, followed by basal cell proliferation [79]. Considering that prostatic acini are the androgen-responsive part of the prostatic gland, ductal and urothelial tissue is not affected by castration [34]. Knowledge of the reduction of prostatic size and volume after castration makes reports of a high risk of prostatic neoplasia in castrated dogs a surprising finding. Although different authors provide information on the incidence of castrated dogs in their studied population, no unanimity can be found when considering the impact castration might have on the development of prostatic neoplasia. L'Eplattenier et al. [59] report that castration not only has no effect on the progression of the disease but it does not even prevent the occurrence of prostatic neoplasia. The same may be said about Obradovich et al. [29] and Bell et al. [30] who include castrated animals in their population, yet conclude that an association between castration and prostatic neoplasia cannot be determined. Cornell et al. [32] are the first to hypothesize a potential effect of castration on the progression of the neoplasia from androgen-dependent to an androgen-independent state. They consider neither castration as preventive for the development of prostatic neoplasia nor life-time testis exposure as essential for the development, yet suggest a decreased risk in dogs castrated before the age of six months. In 2002, Teske and co-authors [6] follow and support the hypothesis on the impact of castration on tumor progression in their study conclusions. Bryan et al. [15] find the clearest words by describing a highly significant association between castration and the development of prostatic neoplasia. Nevertheless, they acknowledge the positive impact of castration in terms of lifetime. Considering the lack of information on median survival time, metastatic behavior and other outcome-related factors within the study of Teske et al. [6] and the fact that MST did not differ, or differed in a positive manner between castrated and intact male dogs in other studies, it remains difficult to understand on the basis of which data a favorable influence of castration on tumor progression may have been suggested [6,32]. After a thorough evaluation of the data presented in the various publications, the only conclusion that can be made is that there is no protective effect of castration for the development of prostatic neoplasia. Several aspects should be considered in the evaluations of these studies, such as the differences in study design, the differences in the studied population and most importantly the different definitions on when a dog is to be considered castrated. Although some authors provide information on the CDI within their studied population, either in the form of a mean, median or a range, the minimal CDI necessary to consider an animal castrated at the time of diagnosis is a value mostly neglected. In the few studies in which this minimal CDI is described, the value differs greatly [6,29]. A minimal distance between castration and diagnosis of CPN of 100 days seems to us far too little to ensure the absence of neoplastic cells at the time of castration. We consider the classification used by Obradovich et al. [29] as appropriate, easy to understand and very informative.

We cannot underline enough the importance of such information and it seems inevitable that a minimal CDI should always be reported in future studies on the subject in order to reasonably rule out the possibility that the neoplasia was already present at the time of castration. Castration was and is by most still considered a very important tool in the prevention and long-term treatment of the most common prostatic disorder, BPH [21]. Comparing the incidence of BPH and prostatic neoplasia, it is obvious that clinicians will be more often confronted with BPH and its treatment than with prostatic neoplasia. Shidaifat and co-authors [70] conclude that their results do not agree with the hypothesis that castration plays a role in prostatic neoplasia development. This finding may be considered supported by L'Eplattenier et al. [59], who describe that castration has no effect on tumor progression although gonadectomy has no protective effect against the disorder. On the contrary Heuter et al. [80] acknowledge an increased risk for castrated dogs, yet consider the risk so low that it should be regarded as clinically irrelevant. Obradovich et al. [29] conclude that castration had no influence on the development of prostatic neoplasia in their studied population and Krawiec and Heflin [24] further pointed out that most prostatic disorders, e.g., BPH, may be prevented by surgical castration; a conclusion supported also by Sorenmo et al. [34]. It is mainly Teske et al. [6] and Bryan et al. [15] who consider the impact of castration on prostatic neoplasia important, suggesting castration a possible promoter from an androgen-dependent to an androgen-independent state of a tumor. Bryan et al. [15] describe the association between castration and prostatic neoplasia development as highly significant, yet point out that the higher median age at diagnosis shows a benefit of gonadectomy in terms of lifetime. Although intact male dogs may be affected by numerous prostatic disorders (e.g., prostatitis, BPH, abscess etc.), castrated males are rarely affected by prostatic pathologies. In the rare case of a castrated male presenting with a prostatic disorder, it is significantly more often a CPN rather than any other of the prostatic disorders [39]. Considering all previous reports, the assessment of Polisca et al. [39] can hardly be negated as in fact, prostatic neoplasia is one of the few prostatic disorders that may be found in castrated males. Although these results show that castration has no protective effect against the development of CPN, it may nevertheless be considered proven that there is a beneficial effect of castration on the prevention of other prostatic disorders. Until today, there is neither proof of castration having an impact on the development and/or progression of prostatic neoplasia in a positive or negative fashion, neither is there any scientific evidence that castrated dogs are affected by a more or less aggressive type of prostatic neoplasia. The impact of a gonadectomy on the development of prostatic neoplasia is on the basis of current knowledge that is difficult to assess. We consider it most interesting that, although odds ratios and increased risks may have been statistically proven in different studies [6,29,30,32], only a few authors conclude that there is, in fact, an association between castration and CPN development. Nevertheless, the age of the dog may be taken into consideration when programming an elective gonadectomy. In most studies, dogs affected by CPN are either six years of age or older [1,6,15,23,24,27,30,32]. These reports give reason to believe that castration of adult male dogs under the age of six years may be performed with a very low risk of concomitant presence of CPN, whereas castration of dogs over the age of six years may be considered after a thorough evaluation of the prostate. The same may be said on immunohistochemical findings and findings of metastatic behavior. It has been reported that positivity for CK-7 was associated with a higher frequency of metastatic disorder (in particular a higher frequency of bone metastasis). Further, dogs with CK-7 positive tumors have been reported to have been castrated at a younger age. Nevertheless, these findings do not provide enough evidence to support the statement that dogs castrated at a younger age suffer from a more aggressive type of CPN, not even for the authors of the study themselves [34]. We consider this caution in drawing conclusions of major importance regarding the possible influence of castration in the context of CPN development. Sadly, this caution is often missing when results are presented and cited in experimental studies as well as in reviews. Unfortunately, it is not uncommon to find citations in review papers (cited in this review) which are not completely in line with what was stated in the cited reference. Furthermore, the presentation of odds ratio and the increased risk is difficult to be interpreted in the context of a scientific review without information on the study

design, inclusion criteria, and conclusions drawn by the authors. Conclusions that may be drawn on the basis of results taken out of context may have an important effect on different stakeholders, e.g., pet owners or veterinary practitioners. Gonadectomy in both male and female dogs has been further linked to other possible side-effects, such as incontinence [81] and obesity [82]. Furthermore, studies have reported an increased risk for the development of osteosarcoma [5], mastocytoma [8], lymphoma [7], hemangiosarcoma [4], and different orthopedic conditions [83]. Similar to the reports on an increased risk of CPN development in the castrated male dog, these studies also have to face important limitations. Some authors limit their evaluation on specific breeds [83,84] which may be a confounding factor for the results and conclusions. Yet the limitation we consider as the most important one is the lack of information on the castration-diagnosis-interval. The majority of studies classifies the population as either spayed and intact [4,7,8,85] or early-spayed, late-spayed and intact [83,84]. The CDI, as has been described above for CPN may be considered to be of the same importance for other neoplastic disorders to suspect or hypothesize a causal relationship between gonadectomy in the dog and cancer development.

9. Conclusions

Within the last two decades, castration was defined as a risk factor not only for prostatic neoplasia but for other neoplastic disorders in both males and females, causing an international discussion if elective gonadectomy in dogs is still a tool that should be used routinely. However, there is currently not enough evidence supporting an increased risk of developing prostatic neoplasia after castration to take a decision against elective gonadectomy in male dogs. More research is needed on this topic and, most importantly, scientific papers on this topic should always provide detailed information on (a) whether or not dogs were castrated, (b) when castration was performed during life, (c) the size of the reference population, (d) how long before the diagnosis the dog was castrated in order to be included in the castrated group. In healthy dogs, gonadectomy should be considered a reliable and valuable preventive treatment for BPH and other non-malignant prostatic disorders. On the basis of the data provided by the currently available scientific literature, elective gonadectomy of adult male dogs of under six years of age cannot be excluded from the daily veterinary practice due to concern of causing prostatic neoplasia until clear and strong evidence is available.

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5.3. Appendix 3: List of Abbreviations

Abbreviations Part 1:

pO_2	partial pressure of oxygen
SO_2	oxygen saturation
pCO_2	partial pressure of carbon oxide

Abbreviations Part 2:

HAS	hemangiosarcoma
MCT	mast cell tumor
CPN	canine prostatic neoplasia
HD	hip dysplasia
CCT	cranial crucial ligament tear
BCS	body condition score
CDI	castration diagnosis interval
CEH	cystic endometrial hyperplasia
BPH	benign prostatic hyperplasia
PLGH	percentage of lifetime exposure to gonadal hormones
AAC	age at castration

Abbreviations Part 3 and 4:

ERβ	estrogen receptor beta
ERα	estrogen receptor alpha
PCR	
GPER	G-protein coupled estrogen receptor
VSMC	vascular smooth muscle cells
PR	progesterone receptor
ER	estrogen receptor
IHC	immunohistochemistry
GnRH	gonadotropin-releasing hormone
FSH	follicle stimulating hormone
LH	luteinizing hormone
Ab	Antibody
NBF	neutral buffered formaldehyde