Expression of the insulin-like growth factor (IGF) system and steroidogenic enzymes in canine testis tumors

Monique AJ Peters1,4, Jan A Mol1, Monique E van Wolferen1, Marja A Oosterlaken-Dijksterhuis1, Katja J Teerds2,3 and Frederik J van Sluijs*1

Address: 1Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Universiteit Utrecht, The Netherlands, 2Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Universiteit Utrecht, The Netherlands, 3Department of Animal Sciences, Human and Animal Physiology Group, Wageningen University, The Netherlands and 4Centre for the Quality of Care, University of Nijmegen, The Netherlands

Email: Monique AJ Peters - majpeters@wish.net; Jan A Mol - j.mol@vet.uu.nl; Monique E van Wolferen - M.E.vanWolferen@vet.uu.nl; Marja A Oosterlaken-Dijksterhuis - m.a.oosterlaken-dijksterhuis@vet.uu.nl; Katja J Teerds - katja.teerds@alg.fmd.wau.nl; Frederik J van Sluijs* - F.J.vanSluijs@vet.uu.nl

* Corresponding author

Abstract

Testis tumors occur frequently in dogs. The main types of tumors are Sertoli cell tumors, seminomas, and Leydig cell tumors. Mixed tumors and bilateral occurrence of tumors may be encountered frequently. To elucidate the possible relationship between the insulin-like growth factor (IGF) system and the development of different types of testis tumors in dogs, the expression of insulin-like growth factor-I and II (IGF-I and IGF-II), their type I receptor (IGF-IR), and their binding proteins (IGFBPs) was examined. In addition the expression of the steroidogenic enzymes p450-aromatase and 5α-reductase type I and type II, and the androgen receptor (AR) was investigated by a semiquantitative reverse-transcriptase PCR (RT-PCR). Both normal testes and testes with tumors were studied. In normal testes a clear expression of IGF-I, IGF-II, IGF-IR, IGFBP2, IGFBP4 and IGFBP5 was found. Expression of IGFBP1 and IGFBP3 was weak. There was also clear expression of the steroidogenic enzymes 5α-reductase, aromatase, and the AR. Quantification of RT-PCR products revealed significantly less expression of IGFBP1, IGF-I, and 5α-reductase type I in Sertoli cell tumors and seminomas. Leydig cell tumors and mixed tumors had a significantly higher expression of IGFBP4 and IGF-IR than normal testes. The expression of aromatase was lower in seminomas and in mixed tumors. The expression of AR, IGF-II and IGFBP2, IGFBP3, IGFBP5, and 5α-reductase type II did not differ among the different types of tumors. It was concluded that Sertoli cell tumors and seminomas have a comparable expression of the IGF system while Leydig cell tumors have a different pattern, suggesting difference in pathobiology among these types of tumors.

Background

The prevalence of testicular tumors is higher in dogs than in any other species of domestic animals and higher than in humans. Especially in older dogs the prevalence can be as high as 60% [1]. Testis tumors in dogs seldom metastasize and can be considered to be benign proliferations [2]. The three main types of testis tumors in dogs are Sertoli cell tumors, seminomas, and Leydig cell tumors and...
combinations of them occur often. These tumors are seldom lethal, but can cause feminization of the dog, which in severe cases can lead to a fatal bone marrow depression [2]. Feminization is caused by hyperestrogenism and is mostly associated with Sertoli cell tumors but Leydig cell tumors and seminomas have also been associated with this syndrome. When feminization occurs in dogs with a seminoma, it is presumed that a co-existing Sertoli or Leydig cell tumor is responsible for the hyperestrogenism [2].

There is evidence that growth factors of the insulin-like growth factor regulatory system, such as insulin-like growth factor-I (IGF-I) and IGF-II, are involved in the pathobiology of neoplasia, both in terms of the risk of developing a tumor and in terms of its behavior [3].

Insulin-like growth factor-I (IGF-I) and IGF-II are peptides believed to play an important role in the regulation of cellular growth and differentiation. The IGFs are synthesized and secreted by many tissues. They can act as endocrine hormones that are being transported by the circulation to distant sites of action, but they can also act locally by paracrine or autocrine mechanisms. The biological activity of IGF-I and IGF-II is modulated by their binding proteins and receptors. Two distinct receptors and six different high-affinity binding proteins have been identified [4,5].

Both IGF-I and IGF-II are produced locally in the testis [6–9]. The IGF system plays an important role in the local regulation of testicular function [10]. IGF-I has different roles in the two major compartments of the testis, the interstitium containing the Leydig cells and the seminiferous tubules containing Sertoli and germ cells. In Leydig cells it stimulates testosterone synthesis [11], in spermatogonia it is involved in stimulation of DNA synthesis [12], while in Sertoli cells it stimulates lactate synthesis [13] and glucose transport [14]. IGF-II has a clearly established role in embryonic and fetal development, but its postnatal function remains unclear [15] although culture experiments have shown that it can stimulate spermatogonial proliferation [12].

Two major gene classes, the proto-oncogenes and tumor suppressor genes, can trigger tumor formation after becoming mutated. It is known that components of the IGF system are upregulated by oncogenes, while tumor suppressor genes can inhibit this system [16]. Hence, mutated proto-oncogenes may induce the abundant synthesis of growth factors like the IGFs [17]. Subsequently, autocrine regulation of growth can occur in a number of tumors, implying that the tumor cells have gained the ability to grow autonomously [18]. Since mixed tumors are encountered frequently in the canine testis, one could argue that both autocrine and paracrine mechanisms may play a role in tumor induction [19]. This could raise the hypothesis that neighboring cells following several mutations could be triggered to transform and become neoplastic even if they are of different origin, leading to formation of different types of tumors in one testis.

When investigating a model for carcinogenesis, testis tumors could be considered as hormone-related cancers whereby hormones drive cell proliferation. Genes involved in steroid hormone metabolism and transport are of interest. We investigated four genes: p450-aromatase, the androgen receptor and 5α-reductase type I and II. The p450-aromatase (P450-aro) enzyme converts testosterone to estrogens, the androgen receptor is responsible for androgen transport and 5α-reductase is responsible for the conversion of testosterone to the metabolically more active dihydrotestosterone (DHT). Two isoforms of 5α-reductase have been identified and are referred to as type I and type II. The formation of DHT is IGF-I dependent [20].

Because tumors occur more frequently in the canine testis than in any other species, we hypothesize that the IGF-system is stimulated in the testis of the aging dog. To test this hypothesis we examined the gene expression of IGF-I, IGF-II, and IGF-IR and the binding proteins IGFBP1 to IGFBP5 in Sertoli cell tumors, seminomas, Leydig cell tumors, and mixed tumors. Furthermore, we also studied the expression of the enzymes p450-aromatase and 5α-reductase (type I and II) and the androgen receptor, which may be indicative of differentiation induced by the IGF system.

Materials and Methods

Material

Twenty-four dogs with testis tumors and 6 dogs with normal testes were studied. Approval of the ethical committee of the University of Utrecht for the use of the animals for research was obtained. The testes were collected from several veterinary clinics and a high number of different breeds of dogs were included in this study. The age of the dogs varied from 3 to 14 years. Bilateral orchidectomy was performed except in 2 dogs in which only one testis was present. Four dogs had signs of feminization. Tumors were dissected free as well as possible, cut in pieces, and immediately placed in liquid nitrogen. One piece of tumor material was fixed by immersion in Bouin’s solution for at least 24 hours. If a (macroscopically) nonhomogeneous tumor was present, more pieces of tissue were fixed. The frozen pieces were stored at -70°C until further processing.

Characterization of tumors

The pieces of tissue fixed by immersion were embedded in paraffin and sections were cut at 5 μm. Sections were stained with Mayer’s hematoxylin and eosin for
histological examination. Paraffin sections were also stained with an antibody against the LH receptor which is characteristic for Leydig cells in the testis [21]. An antibody against vimentin was used to identify Sertoli cells. This antibody does not stain seminoma cells in dog testes and can, therefore, be used to discriminate between Sertoli cells and seminoma cells [22] as was described in an earlier study [21].

Immunohistochemical staining was also performed with frozen material. Frozen pieces of tumorous testis tissue were cleaved, one half being used to prepare cryostat sections of 5 µm, which were stained with the antibody against LH receptor, and the other half being used to study gene expression.

**Tissue processing for RNA isolation and RT-PCR**

Frozen pieces of testicular and tumorous tissue were weighted. About 1 g of frozen material was transferred to a liquid-nitrogen-cooled container and ground thoroughly with a dismembrator (Braun Biotech Int., Melsungen, Germany) for 45 s at 2200 rpm. Tissue powder was decanted into another liquid-nitrogen-cooled tube and stored at -70°C. When necessary the remaining material was homogenized again for 45 s with the dismembrator. This step was repeated until all pieces were completely ground into powder.

Total RNA isolation was performed using the Qiagen RNAeasy Maxi Kit (Westburg, Leusden, The Netherlands). Reverse transcription of the poly (A)⁺ RNA was performed with a reverse transcription system (Promega Corporation, Madison, WI). Five µg of total RNA was used for this reaction in a mixture of 30 µl 25 mM MgCl₂, 10 µl 10 × AMV-RT buffer, 10 µl 10 mM dNTPs, 100 units RNasin RNase inhibitor, 75 units AMV reverse transcriptase, 2.5 µg oligo (dT) primer, and RNAse free water to a final volume of 100 µl. The reaction occurred for 60 min at 42°C followed by 5 min at 95°C. For the PCR reaction 10 µl of the RT-PCR mixture together with 1.25 units Taq DNA polymerase (Promega), 4 µl 10 × PCR buffer (Promega), and 10 pmol of each primer were combined. Sterile water was added to a final volume of 50 µl. Primers for aromatase, 5α-reductase, androgen receptor, IGF-I, IGF-II, IGF-IR, and IGFBP1 to IGFBP5 were used for the different reactions (Table 1).

A PCR with sterile water with or without RT mixture was used as a negative control. The numbers of cycles were chosen in such a way that the reactions were in the exponential phase of the amplification reactions, enabling a semiquantitative evaluation of the results. For this purpose several tests were carried out with 20, 25, 30, and 35 cycles to estimate the linear section. PCR was performed in a Perkin Elmer Cetus or a MJ Biozym thermal cycler using 30 or 35 cycles of denaturation (94°C, 1 min; first cycle 94°C, 5 min), annealing (55–60°C, 1 min) and extension (72°C, 1 min) followed by a final extension of 10 min at 72°C (Table 2).

Fifteen µl of all PCR products were separated on 1.2% agarose gels stained with ethidium bromide and photographed under UV illumination using a CCD camera. The DNA molecular weight marker VI was used as a size marker (Boehringer, Mannheim, Germany). Photographs were scanned and band intensities were measured using Molecular Analyst software (BioRad Laboratories, Veenendaal, The Netherlands). Fragment sizes were quantified and compared with that of negative controls. Per pair of primers all samples were separated on one gel and each gel contained a negative control.

| Primer | Size | Sense | Antisense |
|--------|------|-------|-----------|
| Aro    | 419  | CCCACTTCAGGTTCCTCTCGATG  | TGTTAGAGGGTGTCAGATG |
| AR     | 598  | CGGTAGGCGACGACCATCGGCGAG | TGCTCCCGCCTGCTACCTT |
| IGF-I  | 560  | ATGGCTCTCGACTCTCGTCTT  | TCGCCCTCATGTTGTCAG |
| IGF-II | 450  | TGGGCGTCTCCTGCTGCTG  | GCAGCTGACCTGTCCT |
| IGF-IR | 437  | AATACGATGCGGAGGTGAGTG  | TGCCCTTGAGAATGTCAT |
| Red 1  | 213  | CTGAGAAATCTCAGAAACCC  | TGTAGTACCAACGGTAT |
| Red 2  | 250  | TCACTTAAGGGAGGCGGTTTC  | ACAAGCCACCTTGGAATC |
| IGFBP1 | 240  | ATACAATCGGAGAAGCTCTG  | TCTGTTGACAGTTTGG |
| IGFBP2 | 553  | AACGCGGAGACGACTCTGA  | AGGCACCGGCTGCTAGT |
| IGFBP3 | 200  | AGTGAGTCCCGAGGAGGACC  | GACTCGAGAGAGAGTCT |
| IGFBP4 | 513  | CAGGGTTGTTTGACCACTTGC  | CAGCATTTGCCACGCTGT |
| IGFBP5 | 484  | GCAGGAGGAGGAGAGCCCGC  | TCCAGCACCCAGGATG |

Aro = p450-aromatase enzyme, AR = androgen receptor, IGF = insulin-like growth factor, IR = receptor type I, Red 1 = type I 5α-reductase enzyme, Red 2 = type II 5α-reductase enzyme, IGFBP = binding protein.
All PCR products were sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). For sequence analysis an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) was used. Sequences were matched with the GenBank using the Blast Search program in order to be certain that the correct products were identified.

Statistical Analysis
Dogs with different types of tumors were compared separately with dogs with normal testes. Dogs with mixed tumors were not evaluated according to the type of tumor but as one group. Differences were evaluated statistically using SPSS 7.5 software. A one-way Anova was used with post hoc tests (LSD) to analyze differences between tumors and normal testes. Differences were considered to be significant when p < 0.05.

Results
Characterization of testis tumors
Immunohistochemical characterization of the tumors with Leydig cell specific antibodies against the LH-receptor and 3β-hydroxy steroid dehydrogenase (3β-HSD) and the Sertoli cell specific vimentin antibody revealed that 7 dogs had Leydig cell tumors, 7 had seminomas, 6 had Sertoli cell tumors and 4 had mixed tumors. The mixed tumors consisted of 2 combinations of Sertoli cell tumor and Leydig cell tumor and 2 combinations of seminoma and Leydig cell tumor. There were bilateral tumors in 9 dogs. Seven of these dogs had identical tumors in both testes. These were Sertoli cell tumors (4 dogs), seminomas (1 dog), Leydig cell tumors (1 dog), and mixed Sertoli cell tumor and Leydig cell tumor (1 dog). Two dogs had a Sertoli cell tumor in the left testis and either a Leydig cell tumor or a seminoma in the right testis.

In 3 dogs with a Sertoli cell tumor and 1 dog with a Leydig cell tumor there were signs of feminization.

Expression of genes
In the normal testes as well as in testis tumors, a clear expression was found by RT-PCR analysis of IGF-I, IGF-II, IGF-R and the binding proteins IGFBP2, IGFBP4, and IGFBP5. The expression of IGFBP1 and IGFBP3 was relatively low or absent (Fig. 1). There was also a clear expression of both types of the 5α-reductase enzyme (types I and II), the androgen receptor, and p450-aromatase (Fig. 1). Compared to normal testes Sertoli cell tumors and seminomas had significantly lower expression of IGFBP1, IGF-I, and 5α-reductase type I. Seminomas also had a significantly lower expression of aromatase (Table 3). No differences were found with regard to the other genes investigated. Thus, except for aromatase the expression patterns of these two different types of tumors were very similar. Compared to normal testes Leydig cell tumors and mixed tumors had a significantly higher expression of IGFBP4 and IGF-IR (Table 3). The expression pattern of the mixed tumors was quite similar to that of the Leydig cell tumors. The expression of aromatase was significantly lower in dogs with mixed tumors than in dogs with normal testes (Table 3).

In three Sertoli cell tumors and one Leydig cell tumor of dogs that showed feminization there was no increase in the aromatase expression.

Discussion
RT-PCR analysis revealed a clear expression of the androgen receptor (AR), aromatase, IGF-I, IGF-II, 5α-reductase type I and II, and IGFBP5 in almost all samples from dogs with testis tumors and from dogs with normal testes. There was a less pronounced but still visible expression in
almost all samples for IGFBP2, IGFBP4, and IGF-1R. In only a few samples there was a clear expression of IGFBP1 and IGFBP3. Care was taken to limit the number of cycles for the RT-PCR in order to remain within the linear part of the amplification reaction. It should be stressed that the results have to be interpreted as semiquantitative and with caution. Nevertheless, using the RT-PCR technology, indications of changes in gene expression patterns can be evaluated.

Earlier studies have shown expression of most genes of the IGF system in mammary gland tissue of dogs [23,24]. To the best of our knowledge, this is the first report of the expression of genes of the IGF system in the canine testis, implicating that this system may play a role in the adult canine testis. In the human and rat testis it has been shown that the IGF system is expressed and plays an important role in testicular functioning [9,25].

In the present study differences in the expression of several genes of the IGF system were observed between normal testis tissue and the different types of testicular tumors. IGF-I and IGFBP1 expression were reduced in Sertoli cell tumors and seminomas. IGFBP4 and IGF-RI mRNA levels were not different from normal dog testes. In contrast to these observations, IGF-I and IGFBP1 mRNA levels were increased in human neoplastic tissues, where the IGF system is believed to play a role in tumorigenesis [26–28]. Although it does not seem likely that the IGF system plays an important role in the development of canine Sertoli cell tumors and seminomas, measurement of IGF, IGF-RI and IGFBP proteins in tumor tissue should be carried out to elucidate a possible role of this system in development of these two types of tumors.

In dogs with a Leydig cell tumor, or mixed tumors with a Leydig cell component, significantly higher IGF-1R and IGFBP4 expression was observed. IGFBP's may compete with the IGF-R's for binding of the available IGF. In cultured porcine Sertoli cells an increase in IGFBP3 has been shown to result in a decrease in bioactive IGF, suggesting that IGF binds with higher affinity to the binding protein than to its receptor [29]. Hence, although IGF-I mRNA levels were not different from normal testes tissue in dogs with Leydig cell tumors and mixed tumors, we cannot exclude that the modulation of IGF-I action may be different among these groups of dogs, due to changes in IGFBP4 and IGF-1R mRNA levels. Moreover, we have only measured the IGF-I, IGF-RI and IGFBP mRNA levels in testes of dog with large tumors. We can not exclude the possibility that the initial growth phase of the tumors coincides with elevated levels of some of the members of the IGF system, followed by a decrease when the tumor ceases to grow or when tumor growth is reduced. This needs to be further investigated.

In general the changes observed in the expression of the IGF system among the different types of tumors is not very dramatic when compared to normal testis tissue. This does not make it likely that this system plays an important role in tumor maintenance and growth. In order to determine whether the IGF system is involved in the initiation of tumor development in the canine testis, mRNA levels should be determined from middle aged dogs to old dogs when the chance of tumor development increases dramatically. IGFs are also known to contribute to the process of tumor metastasis by stimulating the motility of malignant cells [30]. Since testicular tumors in dogs are usually not malignant [2], it could be argued that the relatively normal expression levels of the IGF system contributes to the low tendency to metastasize in this species.

The lower expression of 5α-reductase type I in dogs with Sertoli cell tumors and seminomas compared to dogs with
normal testes, suggests a reduced capacity to form androgens. The 5α-reductase enzymes type I and type II which convert testosterone to the metabolically more active form dihydrotestosterone (DHT), are localized in testicular tissues [31,32]. Since the conversion of testosterone in DHT is IGF-1 dependent [20]. The reduction of 5α-reductase expression in Sertoli cell tumors could be a result of the lower IGF-1 expression. In human skin fibroblasts an increase in IGF-I levels has been shown to cause a rise in 5α-reductase activity [33]. It is not likely that the seminoma cells themselves express 5α-reductase, hence, the decreased 5α-reductase expression in these tumors is presumably the result of the reduced number of Sertoli cells and Leydig cells present in the tumor.

Seminomas and mixed tumors had a lower expression of aromatase than normal testes tissue, while the expression in Sertoli cell tumors and Leydig cell tumors was comparable to that in normal testes even when signs of feminization were present. A comparable expression of aromatase in these tumors was a rather unexpected finding, since the presence of both Sertoli cell tumors and Leydig cell tumors in dogs can result in elevated estradiol levels, sometimes even causing feminization [34]. In other mammals, including man, aromatase has been found in Leydig and Sertoli cells as well as in germ cells [35], while in the equine testis only Leydig cells have been shown to contain aromatase activity [36]. There are several possible explanations why aromatase expression in dogs with Sertoli cell tumors and Leydig cell tumors is comparable to that in normal dogs, although the level of estradiol in peripheral and testicular venous blood was elevated [37]. First, the aromatase enzyme is not the rate-limiting step in the formation of estrogens unless it becomes saturated. A condition that is very unlikely to happen since the level of testosterone in dogs with Sertoli cell tumors is very low [37]. Second, due to the reduced 5α-reductase type I expression, there is more testosterone substrate available for the aromatase enzyme to convert to estradiol, since both enzymes use androgens as substrate. Third, there may be no correlation between aromatase activity and thus estrogen synthesis, and aromatase mRNA expression in canine testicular tumors, as was demonstrated in the epididymis of monkeys [38]. Fourth, the level of the catabolic 17beta-HSD enzyme, that is able to convert estradiol to estrone, can become down-regulated in pathological testis tissue leading to an accumulation of estradiol. This issue obviously needs more research in order to clarify the discrepancy between aromatase mRNA expression and estradiol levels in serum.

We did not observe differences in the expression of the androgen receptor among the different types of tumors. The androgen receptor has been implicated to play a role in neoplastic transformation of the prostate in men [39] and by its presence in neoplastic germ cells, a role in the pathogenesis of male germ cell tumors has also been suggested [40]. Expression of the androgen receptor in the canine testis has been examined in a previous study. These authors were unable to detect androgen receptors in benign Leydig cell tumors, while malignant Sertoli cell tumors and seminomas were characterized by higher androgen receptor levels [41]. We investigated only benign tumors, which may explain the differences between our findings and the study mentioned above.

In conclusion, the expression of genes of the IGF system has some different features in canine testis tumors compared to normal testis tissue. Similar expression patterns for IGF-I and IGFBP1 were found for Sertoli cell tumors and seminomas, suggesting the possibility of a common pathobiology. Leydig cell tumors and mixed tumors, all of which contained Leydig cell tumor components had a different expression pattern, in these tumors the expression of IGF-1R and IGFBP4 was increased. However, the observed changes in gene expression were all relatively

### Table 3: RT-PCR analysis of canine testis tumors

| Diagnosis     | ARO     | IGFBP1 | IGFBP4 | IGF-I | IGF-1-R | RED 1 |
|---------------|---------|--------|--------|-------|---------|-------|
| SCT (n = 15)  | 20.4 ± 8.8 | 10.7 ± 5.4* | 17.9 ± 12.2 | 26.3 ± 12.1* | 5.6 ± 2.2 | 41.3 ± 23.3* |
| SEM (n = 7)   | 17.0 ± 2.9* | 8.7 ± 6.5* | 17.0 ± 15.1 | 27.1 ± 9.0* | 10.4 ± 4.1 | 38.3 ± 17.9* |
| LCT (n = 9)   | 19.4 ± 10.6 | 16.0 ± 4.9 | 33.3 ± 18.5* | 35.4 ± 11.5 | 21.3 ± 3.2* | 66.7 ± 20.6 |
| MIXED (n = 5) | 13.4 ± 5.3* | 20.8 ± 9.4 | 36.8 ± 17.3* | 30.8 ± 10.4 | 22.3 ± 8.4* | 66.6 ± 6.7 |
| NORMAL (n= 13)| 27.6 ± 2.8 | 17.4 ± 9.4 | 19.5 ± 11.3 | 37.3 ± 9.3 | 7.9 ± 1.2 | 69.0 ± 15.9 |

SCT = Sertoli cell tumor, SEM = seminoma, LCT = Leydig cell tumor, mixed = a combination of testis tumors, ARO = p450-aromatase enzyme, IGFBP = insulin-like growth factor binding protein, IGF = insulin-like growth factor, IGF1-R = insulin-like growth factor receptor and RED 1 = 5α-reductase type I. The number of tumors included in the measurements is indicated between brackets. Asterisk indicates significant difference between the tumor and the normal testis (p < 0.05).
small, and thus it does not seem likely that the IGF system is important for tumor maintenance and growth in the dog testis. Whether this system is involved in the initiation of tumor growth is subject to further investigation. Furthermore, signs of feminization cannot be attributed to increased expression of aromatase mRNA levels. In order to obtain more insight in this complicated model of multiple tumor induction in the canine testis, additional research in necessary.

Acknowledgments
We are very grateful to all veterinarians, technicians and assistants who collected the testis tumors. We thank E. Timmermans — Sprang, J. Wolfswin- kel, and M. de Boer-Brouwer for technical assistance. Many thanks to Dr. E. van Garderen, Utrecht University, for the sequence of the IGF-I and IGF-II, and Dr. Paul Span, University of Nijmegen, for the sequence of the can- nine-specific Sx-reductase primers. The critical reading of the manuscript by Dr. B. Belshaw is highly appreciated.

References
1. Mosier JE 
Effect of aging on body systems of the dog. The Veterinary Clinics of North America 1989, 19:1-17
2. Nielsen SW and Kennedy PC 
Tumors of the genital systems. In: Tumors in Domestic Animals (Edited by: Moulton JE) Berkeley University of California Press 1990, 479-517
3. Rosen CJ and Pollak M 
Circulating IGF-I: New Perspectives for a New Century. Trends Endocrinol Metab 1999, 10:136-141
4. Lowe WJ 
Insulin-like Growth Factors. Scientific American. Science & Medicine 1996, 62-71
5. Clemons DR 
Role of insulin-like growth factor binding proteins in controlling IGF actions. Mol Cell Endocrinol 1998, 140:19-24
6. Casella SJ, Smith EP, van Wyk JJ, Joseph DR, Hynes MA, Hoyt EC and Lund PK. Isolation of rat testis cDNAs encoding an insulin-like growth factor I precursor. DNA 1987, 6:325-330
7. Cailléau J, Vermeire S and Verhoeven G 
Independent control of the production of insulin-like growth factor I and its binding protein by cultured testicular cells. Mol Cell Endocrinol 1990, 69:79-89
8. Koike S and Nomura T 
Immunochemical localization of insulin-like growth factor-II in the perinatal rat gonad. Growth Regul 1995, 5:185-189
9. Zhou Y and Bandy C 
The anatomy of the insulin-like growth factor system in the human testis. Fertil Steril 1993, 60:897-904
10. Rappaport MS and Smith EP 
Insulin-like growth factor (IGF) binding protein 3 in the rat testis: follicle-stimulating hormone and testosterone regulate the distribution pattern of IGFBPs synthesized by the canine mammary tumor cell line CMT-1A. Breast Cancer Res Treat 1997, 48:1-23
11. Lin T, Haskell J, Vinson N and Terracio L 
Direct stimulatory effects of insulin-like growth factor-I on Leydig cell steroidogenesis. In primary culture. Biochem Biophys Res Commun 1986, 137:950-956
12. Soder O, Bang P, Wahab A and Parvinen M 
Insulin-like growth factors selectively stimulate spermatogonial, but not meiotic, deoxyribonucleic acid synthesis during rat spermatogenesis. Endocrinology 1992, 131:2344-2350
13. Oonk RB and Grootegoed JA 
Insulin-like growth factor I (IGF-I) receptors on Sertoli cells from immature rats and age-dependent testicular binding of IGF-I and insulin. Mol Cell Endocrinol 1988, 55:33-43
14. Oonk RB, Jansen R and Grootegoed JA 
Differential effects of follicle-stimulating hormone, and insulin-like growth factor I on hexose uptake and lactate production by rat Sertoli cells. J Cell Physiol 1989, 139:210-218
15. Holly JMP. 
The IGF-2 enigma. Growth Hormone and IGF Research 1998, 8:183-184
16. Baserga R, Hong A, Rubini M, Prisco M and Valentinis B 
The IGF-I receptor in cell growth, transformation and apoptosis. Bio- chim Biophys Acta 1997, 1332:F105-F126
17. Weinberg RA 
How cancer arises. Sci Am 1996, 275:62-70
18. Drescher B, Lauke H, Hartmann M, Davidoff MS and Zunkeller W 
Immunohistochemical pattern of insulin-like growth factor (IGF) I, IGF II and IGF binding proteins 1 to 6 in carcinoma in situ of the testis. Mol Pathol 1997, 50:298-303
19. Dow C 
Testicular tumours in the dog. Journal of Comparative Pathology 1962, 72:247-265
20. Mose S, Di Silverio F, Leonzio S, Varasano P, Martini C, Tosti-Croce C and Sciarra F 
Insulin-like growth factor-I and -II in human benign prostatic hyperplasia: relationship with binding proteins 2 and 3 and androgens. Steroids 1998, 63:362-366
21. Peters MAJ, Teerds KJ, Vanderslagh I, De Rooij DG and van Sluijs FJ 
Use of Antibodies against Luteinizing Hormone Receptor, 3-Hydroxy Steroid Dehydrogenase, and Vimentin to characterise different types of Testicular Tumours in Dogs. Reproduction february 2001
22. Franke WW, Grund C and Schmid E 
Intermediate-sized filaments present in the vimentin type. Eur J Cell Biol 1979, 19:269-275
23. Mol JA, Selman PJ, Sprang EPM, van Neck JW and Oosterlaken-Dijksterhuis MA 
The role of progestins, insulin-like growth factor (IGF) and IGF-binding proteins in the normal and neoplastic mammalian gland of the bitch. A review. Journal of Reproduction and Fertility Supplement. 1997, 51:339-344
24. Oosterlaken-Dijksterhuis MA, Kwant MM, Slob A and Mol JA 
IGF-I and retinoic acid regulate the distribution pattern of IGF-BPs synthesized by the canine mammary tumor cell line CMT-1A. Breast Cancer Research and Treatment 1999, 54:15-23
25. Smith EP, Dickson BA and Chernaeskau SD 
Insulin-like growth factor binding protein-3 secretion from cultured rat sertoli cells: dual regulation by follicle stimulating hormone and insulin-like growth factor-I. Endocrinology 1990, 127:2744-2751
26. Kiess W, Koepf G, Christiansen H and Blum WF 
Human neuroblastoma cells use either insulin-like growth factor-I or insulin-like growth factor-II in an autocrine pathway via the IGF-I receptor: variability of IGF, IGF binding protein (IGFBP) and IGF receptor gene expression and IGF and IGFBP secretion in human neuroblastoma cells in relation to cellular proliferation. Regul Pept 1997, 72:19-29
27. Rosen N, Yee D, Lippman ME, Paik S and Cullen KJ 
Insulin-like growth factors in human breast cancer. Breast Cancer Res Treat 1991, 18(Suppl 1):S55-62
28. Werner H 
Dysregulation of the type I IGF receptor as a paradigm in tumor progression. Mol Cell Endocrinol 1998, 141:1-5
29. Besser V, Le Magueresse-Battistoni B, Collecte J and Benahmed M 
Tumor necrosis factor alpha stimulates insulin-like growth factor binding protein 3 expression in cultured porcine Sertoli cells. Endocrinology 1996, 137:296-303
30. Daughaday WH and Deuel TF 
Tumor secretion of growth factors. Endocrinol Metab Clin North Am 1991, 20:539-563
31. Mahony MC, Swanlund DJ, Bileuter M, Roberts KP and Pryor JL 
The regional distribution of 5alpha-reductase type 1 and type 2 mRNA along the nonhuman primate (Macaca fascicularis) epididymis. J Androdal 1996, 98:1116-1121
32. Mahony MC, Heikinheimo O, Gordon K and Hodgen GD 
The regional distribution of 5alpha-reductase type 1 and type 2 mRNA along the nonhuman primate (Macaca fascicularis) epididymis. J Androdal 1997, 18:595-601
33. Horton R, Pasupuletti V and Antonipillai I 
Androgen induction of steroid 5 alpha-reductase may be mediated via insulin-like growth factor-I. Endocrinology 1993, 133:447-451
34. Rijnbark A 
Testes. In: Clinical Endocrinology of Dogs and Cats (Edited by: Rijnbark A) Dordrecht Kluwer Academic Publishers 1996, 119-130
35. O'Mearcagh AS, Ledger GA, Roche PC, Parisi JE and Zimmerman D 
Aromatase expression in human germinomas with possible biological effects. J Clin Endocrinol Metab 1995, 80:3763-3766
36. Eisenhauer KM, McCue PM, Nyden DK, Osawa Y and Roser JF 
Localization of aromatase in equine Leydig cells. Domestic Anim Endocrinology 1994, 11:291-298
37. Peters MAJ, de Jong FH, Teerds KJ, De Rooij DG, Dielemann SJ and van Sluijs FJ 
Ageing, testicular tumours and the pituitary-testis axis in dogs. J Endocrinol 2000, 166:153-161
38. Pereyra-Martinez AC, Roselli CE, Stadelman HL and Resko JA 
Cytochrome P450 aromatase in testis and epididymis of male rhesus monkeys. Endocrine 2001, 16:15-19
39. Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ and Sugimura Y. The endocrinology and developmental biology of the prostate. Endocr Rev 1987; 8:338-362
40. Rajpert-De ME and Skakkebaek NE. Immunohistochemical identification of androgen receptors in germ cell neoplasia. J Endocrinol 1992, 135:R1-R4
41. Golubeva VA, Kuz'mina ZV, Gershtein ES, Ponomar'kov VL, Bassalyk LS and Blinnikova MG. Steroid hormone receptors in spontaneous testicular tumors in dogs. Vopr Onkol 1992, 38:464-469