Active immunisation against gonadotropin-releasing hormone, an active tool to block the fertility axis in mammals

Johan A. Turkstra and Rob H. Meloen

Introduction

Fertility axis regulation by GnRH

Gonadotropin-releasing hormone (GnRH), also known as luteinising-hormone releasing hormone (LHRH) is produced in neurons in the hypothalamus. It is transported via axons to small blood vessels (portal vessels) in the median eminence, where it is released into the blood. The blood vessels are draining the anterior pituitary, allowing GnRH to reach the anterior pituitary in high concentrations. In the pituitary, GnRH binds to the GnRH receptors on the gonadotropic cells to stimulate the release of follicle-stimulating hormone (FSH) and luteinising hormone (LH) to the circulation. The pulsatile secretion pattern of GnRH induces the cyclic release of LH and to a lesser extent of FSH.

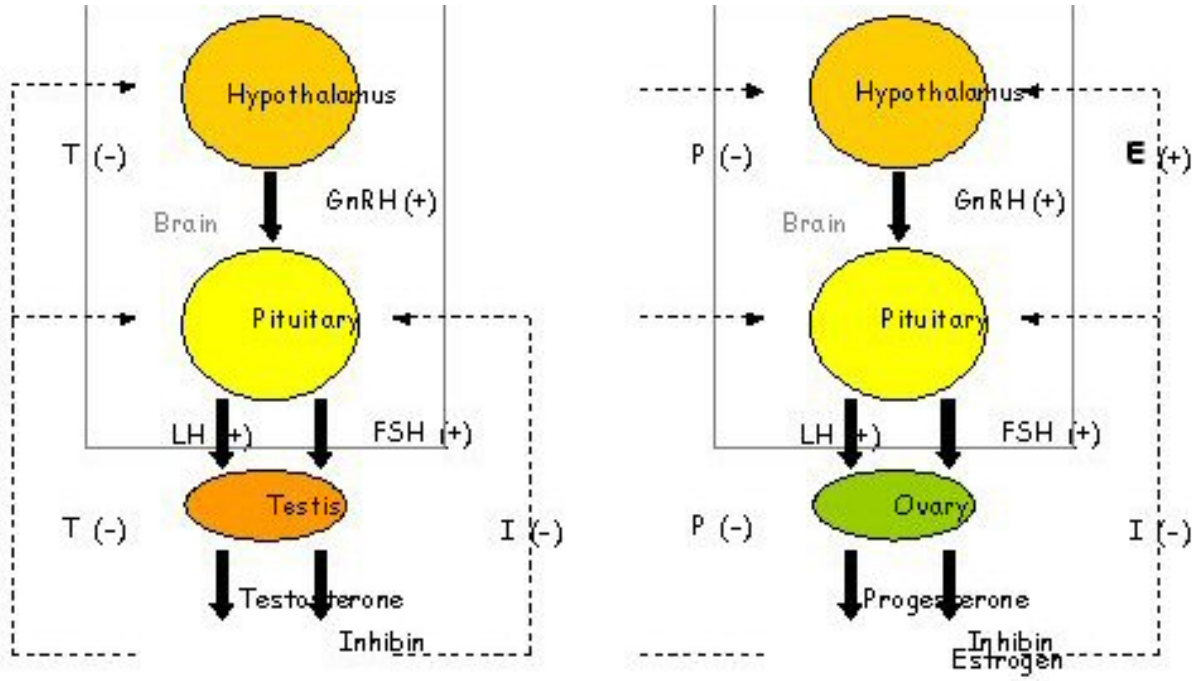

Figure 1. Schematic overview of the fertility axis in male (left) and female (right) mammals. GnRH, LH and FSH are responsible for secretion of testosterone (T), progesterone (P), oestrogen (E) and inhibin (I). High levels of T, P and I, inhibit either GnRH, LH or FSH secretion via a negative feedback. High levels of estrogens are responsible for a positive feedback on GnRH at time of proestrus, subsequently leading to the LH surge and ovulation.

In male mammals, LH stimulates the synthesis and secretion of androgens (e.g. testosterone) from the Leydig cells in the testes. High levels of androgens, directly and indirectly inhibit LH secretion by a feed-back mechanism acting on the pituitary and hypothalamus, where it inhibits GnRH release. FSH is responsible for the initiation of spermatogenesis, whereas in adults it may play a role, together with testosterone, in maintaining sperm production. FSH binds to specific receptors on the Sertoli cells to stimulate the production of many growth factors and other factors such as androgen-binding protein (ABP). ABP is necessary to maintain a high concentration of testosterone in the seminiferous tubules by binding this androgen. Inhibin, also secreted by the Sertoli cells is the major feedback regulator of FSH release. The production and secretion of inhibin is regulated by FSH.

In female mammals, FSH induces follicle growth and subsequently estradiol and inhibin secretion by the granulosa cells. High estradiol levels, produced by mature follicles, lead to a positive feedback on the hypothalamus at time of proestrus, which causes the LH surge responsible for ovulation. During this period, inhibin is secreted by the granulosa cells, causing an inhibition of FSH release. After ovulation the luteinised granulosa and the theca cells start to produce high levels of progesterone, which in turn inhibits LH and FSH secretion from the pituitary.

Targeting GnRH to manipulate the fertility axis

Manipulation of the fertility axis by targeting GnRH has potentials as a tool to block gonadal function in male and female mammals, in order to delay puberty, prevent sexual and aggressive behaviour and sex odours, to establish infertility or to treat reproduction-related diseases. Several approaches may be appropriate to achieve this, like treatments with GnRH agonists or antagonists, passive or active immunisation against GnRH or the use of GnRH targeted toxins.

GnRH agonists

GnRH is a peptide of 10 amino acids with the following amino acid sequence in 3-letter code: pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-amide.
Soon after the discovery of GnRH, the peptide was used to treat infertility [29].Administration of low doses of GnRH in a pulsatile manner stimulates the pituitary-gonadal axis similar to endogenous GnRH. However, chronic administration at higher doses has an inhibitory effect on the pituitary-gonadal axis. Initially LH and FSH secretion are stimulated, this is the so-called flare-up, but due to down–regulation and desensitisation of the GnRH receptors it eventually leads to suppression of pituitary-gonadal function. GnRH agonists with an improve half-life have been developed, leading to GnRH super agonists. Due to their inhibitory effects on the pituitary-gonadal axis, these GnRH super agonists have been used successfully for treatment of reproduction-related diseases, such as precocious puberty [29], endometriosis [29], uterine fibroids [29] and malignancies which are dependent on gonadal steroids, such as prostate cancer [29] and breast cancer [29].

In the veterinary field, however, results are inconsistent. GnRH agonists can be used to prevent ovulation in female cattle, while in male cattle they have a stimulatory effect on gonadal activity [29]. In stallions, suppression of gonadotropins was reported after a single GnRH implant [29], while others found a stimulatory effect on sexual behaviour and sperm quality [29]. In mares GnRH agonist treatment extended oestrus intervals, but did not affect ovarian function [29]. In other species, agonist treatment totally suppressed gonadal activity, for instance in male and female pigs [29], and male and female dogs [29].

GnRH antagonists

The mechanism of action of GnRH antagonists is based on the permanent occupation of the GnRH receptor, which prevents endogenous GnRH to bind to its receptor [29]. In contrast to agonists, GnRH antagonists do not induce an initial stimulating effect, but directly inhibit LH and FSH secretion. The therapeutic applications of GnRH antagonists are the same as those of the agonists, although the lack of a flare-up response may be advantageous in case of short term treatment, such as in vitro fertilisation or treatment of metastasised prostate tumours [29]. The GnRH antagonists Ganirelix and Cetrorelix are currently available for inhibition of premature LH surges in women undergoing ovarian hyperstimulation [29]. The antagonist Abarelix was only conditionally approved for treatment of prostate cancer in men for whom agonist therapy may not be appropriate. Clinical trials which should lead to approval of the drug revealed, however, that Abarelix could induce immediate allergic reactions, including hypotension and unconsciousness, requiring complicated administration procedures [29].

In animals, GnRH antagonist treatment has given variable results. In female cattle, it reduces hormone levels and causes a delay in ovulation [29], whereas others observed only a short suppressive effect on gonadotropin levels [29]. In female pigs, the LH surge and ovulation were blocked by antagonist treatment, whereas FSH and oestradiol secretion were not affected [29]. Total suppression of testosterone and reduction in testis size were observed in young boars given a high dose of GnRH antagonist for 3 weeks [29]. In stallions, antagonist treatment suppressed hormone levels and libido [29], while in mares effects were variable [29]. In general, application of GnRH antagonists in animal species is limited due to the high costs of the treatment and the inconsistent effects.

Active immunisation against GnRH

Antibodies raised against synthetic GnRH peptide vaccines, neutralise endogenous GnRH. This results in infertility in both male and female mammals [29]. In males, inhibition of pituitary secretion of LH and to a lesser extent FSH, results in testosterone deprivation, which subsequently leads to

impaired spermatogenesis, decreased testis size and affected male behaviour. In females, the lack of gonadotropins causes reduced gonadal steroid levels, reduced follicular growth, inhibition of ovulation, and reduced size of ovaries and uterus. In male pigs, GnRH immunisation has successfully been applied as an alternative for surgical castration to prevent the occurrence of boar taint in pork [6] [21]. Boar taint is mainly caused by accumulation of two compounds in the fat tissue; androstenone, which is a gonadal steroid, and skatole, a product of intestinal degradation of the amino acid tryptophane. The presence of both compounds in adipose tissue depends on the sexual maturity of the boar. In humans, GnRH immunisation has been studied for treatment of diseases which are driven by gonadal steroids, such as prostate cancer [25] [28]. Application of immunisation against GnRH has been described in many other species.

Passive immunisation against GnRH

Active immunisation against GnRH requires at least several weeks before an effective GnRH antibody titre is generated. In contrast, passive immunisation results in a GnRH immunisation effect within 24 hours [29]. This technique has been very helpful in studying the pituitary-gonadal axis and the role of the hormones involved. For practical application, however, this approach is not suitable as large amounts of antisera and frequent administration are required to reduce hormone levels [29].

GnRH targeted toxins

Another approach to manipulate the pituitary-gonadal axis is to destroy GnRH receptor bearing cells using GnRH coupled to cytotoxins. Once GnRH has bound to its receptor, the conjugate is internalised, where the toxin than can inhibit protein synthesis, eventually leading to cell death. GnRH conjugated to pokeweed antiviral protein (PAP) proved to be cytotoxic in prostate cancer cell lines [29]. In dogs, a single treatment reduced testosterone levels to zero for approximately 20 weeks. Thereafter, testosterone returned to normal levels, indicating reversibility [29]. Furthermore, little toxicity to normal intact tissue has been reported in animal studies [29]. This approach has been approved by the US Food and Drug Administration for other target molecules involved in cancer treatment [29].

Active immunisation against GnRH in mammals

Vaccine design

Vaccination against the hormone GnRH blocks the hypothalamic-pituitary-gonadal axis. Therefore, it can be used as an alternative for castration and fertility control. This has been described for farm animals [6] [14] [29], companion animals [24][29] and wildlife species [22] [29]. Application of GnRH vaccination in humans has been described for controlling fertility-related endocrine disorders and gonadal steroid-dependent diseases [13] [25] [29]. During the past 3 decades, several attempts have been made to develop a vaccine against GnRH. Successful vaccination against an endogenous molecule requires a sufficient level of neutralising antibodies during the full treatment period, to obtain the desired effect. GnRH, an endogenous molecule, is a small peptide, which consists of only 10 amino acids. Raising an immune response against such a small endogenous peptide, which is not immunogenic by itself, requires more than a standard approach. This means that coupling of GnRH to a carrier protein and the use of a strong adjuvant are not sufficient. Due to these limitations, the efficacy of the vaccine is likely to be insufficient to obtain a full response, i.e. complete cessation of gonadal steroid secretion in all individuals treated. Thus, the immunogenicity of the antigen must be amplified in order to develop an effective vaccine. The more so because, the use of strong adjuvant often results in injection-site reactions, which are undesired or even unacceptable in case of animals used for meat production.

Adjuvant

The adjuvants most commonly used in human and veterinary vaccines are oil-based adjuvants and aluminum hydroxide (Alum). Responses to Alum are often low and of short duration. Oil-based adjuvants are effective in generating a high immune response, but may cause inflammatory reactions. Oil-based vaccines mainly consist of an emulsion of oil, either mineral oil or non-mineral oil, and a water phase, which contains the antigen. In general, water-in-oil emulsions give a higher immune response than oil-in-water emulsions. Complete Freund's adjuvant (CFA) is a mineral oil, which forms a water-in-oil emulsion, and contains killed and dried bacteria to stimulate the immune response. This combination induces high antibody responses; because of these characteristics and CFA being one of the oldest adjuvants used, it is ´the golden standard´ among adjuvants. However, due to the inflammatory side effects, which may occur at the site of injection, its use is mostly restricted to immunisation studies in laboratory animals.

Instead of whole bacteria, bacterial compounds such as muramyl dipeptide (MDP), lipopolysaccharide (LPS) or monophosphoryl lipid A (MPL) can be used to stimulate the immune system. Alternative immune stimulating compounds are saponins, i.e. Quil A and the purified QS21 fraction, bacterial DNA, microparticles, Iscoms, liposomes, virus-like particles, block polymers and dimethyldioctadecylammonium bromide(DDA) [26]. In several GnRH immunisation studies, alternatives for CFA have been evaluated. In rats, Alum appeared to be only slightly less effective as compared to CFA [29], however, in cattle CFA was superior to Alum [29]. In cattle, the use of CFA induced a high antibody response but also caused granulomas at the site of injection, whereas DEAE Dextran (glucose polymer derivative) in combination with a mineral oil resulted in similar antibody titres as CFA with less side effects [29]. In a comparative study in mice, rabbits and goats, CFA was compared with Titermax, which contains non-mineral oil and a block polymer and forms a water-in-oil emulsion, and RIBI adjuvant, which contains non-mineral oil with microbial components and forms an oil-in-water emulsion [29]. Titermax induced antibody titres against GnRH equivalent to CFA in all three species investigated, while titres induced by RIBI were substantially lower. Inflammatory reactions induced by Titermax were mild and transient compared to those induced by CFA. In a comparative study with CFA and ISA 51, which forms a water-in-mineral oil emulsion, CFA was superior to ISA 51 with respect to antibody titres and subsequent effects on testosterone levels when tested in sheep [29]. In contrast, others found effective GnRH antibody responses using ISA 51 combined with DDA in baboons [11]. In conclusion, effective antibody titres can be generated with adjuvants other than CFA, however, responses may differ among studies due to differences in target species, number of immunisations, antigen type and dose.

Antigen

Several approaches have been made to increase the immunogenicity of the antigen in order to be able to use an acceptable adjuvant without making concessions to vaccine efficacy.

Carrier proteins

The most often used approach to make a peptide immunogenic, is to couple it to a protein molecule. Commonly used carrier proteins are KLH, TT, DT, OVA, BSA and HSA. The origin of the carrier protein could be of importance for the level of immunogenicity of the conjugate. The use of 'foreign' proteins is expected to result in conjugates with a stronger immune response. For instance, it was shown that OVA is superior to BSA and HSA in bull calves [14] and antibody responses in rats are higher with TT than with thyroglobulin [29]. On the other hand, it was shown [12] that conjugates prepared with the non-mammalian proteins KLH and TT, were less effective in raising high GnRH antibody titres in sheep than conjugates containing equine serum albumin. In general, most exogenous proteins can be used as carriers, although non-mammalian proteins are expected to be more immunogenic.

The chemical linking of the peptide to the carrier protein is mainly done through a carbodiimide coupling [29], which links the peptide to the protein predominantly by carboxyl- and amino groups. Other conjugation reactions are the maleimidosuccinimide coupling which requires an additional cysteine or a sulfhydryl group in the peptide for coupling to an amino group of the protein [21] or the glutaraldehyde coupling which requires an amino- or sulfhydryl group in the peptide [29].

The site of conjugation may determine the efficacy of the immunisation. Conjugation of GnRH to TT via glutamine at position 1, a substituted D-lysine at position 6 or Gly-OH at position 10 was studied [29]. Conjugation via glutamine at position 1 induced a higher GnRH specific antibody response and reduced testosterone levels in rabbits more effectively than conjugation via positions 6 or 10. Substitution of the glycine at position 6 by a D-lysine for conjugation to DT has proven to be an suitable immunogen in several species, including humans [29]. No difference in GnRH antibody response in male sheep was observed, when GnRH was conjugated to KLH via a substituted cysteine either at position 1, 6 or 10 [12]. These results in mice were confirmed [29]. Although the GnRH antibody titres were similar for the 3 conjugates, specificity of the antisera depended on the site of conjugation. Conjugation via cysteine on position 1 resulted in C-terminal directed antibodies, conjugation via cysteine on position 10 generated N-terminal directed antibodies, while conjugation via cysteine at position 6 generated both N- and C-terminal antibodies. In contrast, it was showed that N-terminal conjugation via an cysteine substitution at position 1 resulted in effective immunisation of rats, while conjugation via cysteine substitution at position 10 was not effective [29]. Other groups used native GnRH extended with glycine and cysteine, conjugated to a carrier protein [10] [29] or longer spacer peptides [25] [29]. Thus, it seems that immunisation with GnRH peptides, conjugated to a carrier protein via the N-terminus results in more effective antibody titres than conjugation via the C-

terminus. However, this was not confirmed in all studies and may depend on the chemical approach used and substitution of amino acids required for coupling.

GnRH fusion proteins

To obtain a more defined antigen with a homogenous structure, some research groups focussed on recombinant proteins with multiple GnRH inserts. Female rats and bull calves were vaccinated with purified hybrid GnRH fimbriae from E. Coli [29]. This antigen generated an effective antibody response after two immunisations when administered in an oil-in-water emulsion. A fusion protein containing a domain of Pseudomonas exotoxin A and 12 copies of GnRH, generated high antibody titres in female rabbits and caused atrophy of the ovaries [29]. Two fusion proteins based on ovalbumin and thioredoxin were studied in mice [29]. Both constructs were partially effective, when given separately, while higher responses were observed in animals which received a combination of both proteins. It was shown that immunogenicity increased with the number of GnRH inserts in the ovalbumin fusion protein [33]. Proteins with 7 GnRH inserts performed better than proteins with 4 inserts. A GnRH fusion protein based on leukotoxin administered in a commercially available adjuvant, reduced serum testosterone concentrations to undetectable levels in pigs [29] and in cats [29]. In cattle, 3 vaccinations were required to reduce testosterone concentrations [29]. In conclusion, efficacy of GnRH fusion proteins varies, which may be due to the immunogenicity of the protein or the number of the GnRH inserts in the protein.

Polyvalent GnRH peptides

Repetition of B-cell epitopes may enhance the immunogenicity of the antigen. Therefore, a GnRH-tandem peptide was designed, containing twice the amino acid sequence of endogenous GnRH [21]. This GnRH-tandem peptide conjugated to the carrier protein Keyhole Limpet Hemocyanin (KLH) induced a complete deprivation of serum testosterone in all treated piglets, while native GnRH conjugated to KLH was only partially effective [21]. All piglets treated with the GnRH-tandem conjugate showed a major reduction in testis weight (Figure 2 and 3). However, less than full efficacy was obtained when the number of animals treated increased.

Figure 2. GnRH-immunised (right) and intact male pig (left)

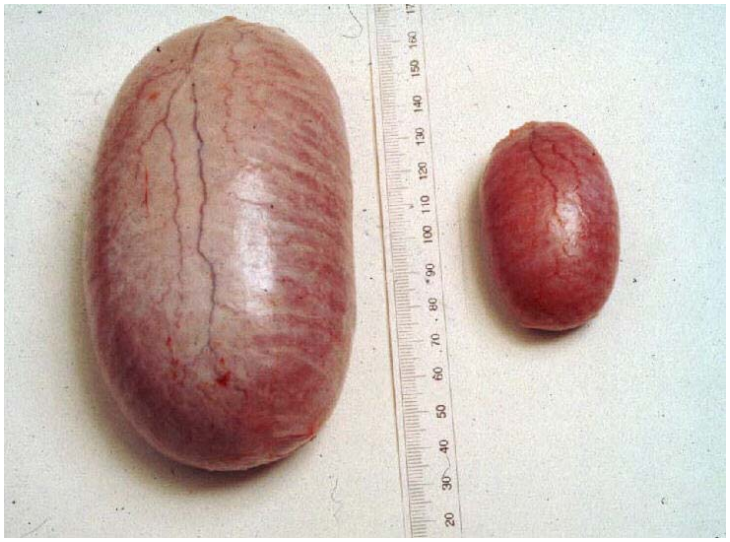

Figure 3. Testis of GnRH-immunised (right) and intact male pig (left).

Others also observed enhanced immunogenicity of dimerised GnRH peptides {29] although the efficacy of the immunisation depended on the site of dimerisation. Dimerisation via a C-terminal cysteine resulted in a dimer which was more immunogenic than its monomeric equivalent when conjugated to a carrier protein, whereas dimerisation via the N-terminal cysteine resulted in a less immunogenic molecule as compared to its monomeric equivalent [29]. Polyvalent constructs can also be generated using the so-called multiple antigenic peptide (MAP) [29]. GnRH-tandem peptides coupled to these lysine-branched constructs and emulsified in CFA, were effective in reducing testis size in male pigs [29].

Synthetic GnRH-T cell epitope constructs and lipopeptides

Completely synthetic peptide antigens containing a T cell epitope and the GnRH decapeptide were tested in several studies, mainly in mice. Mice receiving the GnRH peptide extended with a T cell epitope from the influenza virus hemaglutinin at the N-terminus were infertile for more than 24 weeks after two vaccinations using CFA as adjuvant [29]. The effects of the ligation chemistry used for preparation of the synthetic peptide construct comprising a T cell epitope and GnRH were studied [29]. Linear synthesis, thioether bond formation and oxime bond formation resulted in more immunogenic peptide constructs than peptides linked via a disulfide bond.

In a subsequent study [29], lipids were attached to the GnRH-T cell epitope constructs in order to develop lipopeptides which could be used without an additional adjuvant. The lipids developed in this way were a synthetic version of the N-terminal part of a lipoprotein from gram negative bacteria, dipalmitoyl-S-glyceryl-cysteine (Pam2Cys) and tripalmitoyl-S-glyceryl-cysteine (Pam3Cys). Some of the tested lipopeptides were highly immunogenic and resulted in efficient sterilisation of female mice when administered in saline either via the subcutaneous or intranasal route. In our experiments, a conjugate comprising a GnRH-tandem peptide and Pam3Cys dissolved in PBS was not able to block testis growth in any of the treated male pigs (Turkstra et al., unpublished results). The effects of the nature of the bond between peptide and lipid on the immunogenicity of these constructs were studies [29]. Coupling of a GnRH-tandem peptide to palmitic acid via an a thio-ester bond appeared to be more effective than coupling via an amide bond. The former construct administered in CFA inhibited testis growth in 17 out of 23 treated pigs, while the latter was only effective in 8 out of 20 pigs. In conclusion, GnRH-T cell epitope constructs render mice infertile when administered in CFA, while attachment of lipids, resulted in an immunogenic compound which was effective without an additional adjuvant in mice. In pigs, GnRH lipopeptides require the help of an adjuvant in order to block testis development.

Biological effects of GnRH immunisation

GnRH immunisation has been used to study the effects of hormones involved in the regulation of the hypothalamic-pituitary-gonadal axis. In particular the absence of GnRH at the pituitary site has provided valuable information. Challenge of GnRH immunised animals with GnRH analogs, gonadotropins or gonadal steroids revealed the role of these hormones in pituitary-gonadal function. Effects of GnRH immunisation on pregnancy and reversibility of the effects induced by GnRH

immunisation are also summarised below.

Receptor concentrations and hormone content in GnRH immunised mammals

The role of GnRH in the long-term control of GnRH receptor concentrations was studied by immunoneutralisation of GnRH. In a study in female rats, GnRH immunisation reduced the number of GnRH receptors in the pituitary [29]. A reduction in pituitary GnRH receptor concentration and pituitary GnRH receptor mRNA was also found after passive immunisation of castrated male sheep. This indicates that GnRH stimulation is required to maintain GnRH receptor concentrations [29]. In rhesus monkeys, GnRH immunisation reduced the hypothalamic GnRH content [29]. In contrast, no effects of GnRH immunisation on GnRH content in the median eminence or the body of the hypothalamus in castrated male ponies were observed [29].

Ovariectomized ewes were used to study the effect of GnRH immunisation on pituitary LH and FSH content [1]. The pituitary content of both gonadotropins was significantly reduced by GnRH immunisation. Similar results were found in GnRH immunised castrated male ponies [29] in which LH and FSH content were reduced by 90% and 55%, respectively. It was shown that GnRH immunisation of boars reduced pituitary LH content, but did not affect the FSH content [29]. This is probably due to the fact that in this study, GnRH immunisation did not affect FSH, as FSH levels in circulation were normal. In rhesus monkeys, GnRH immunisation also depressed pituitary LH content [29]. FSH content was not determined in this study. Thus, GnRH immunisation reduces the GnRH receptor concentration and the LH content in the pituitary, while the pituitary FSH content and hypothalamic GnRH content were either not affected or only partially influenced.

Castration of GnRH immunised mammals

Besides the biological effects of GnRH immunisation on hormone levels and gonadal function, efficacy of vaccination can also be determined by surgical castration of immunised animals. Castration of intact male and female mammals results in a so-called post castration rise in LH and FSH, due to the lack of feed back of gonadal steroids. As expected, surgical castration of mammals effectively immunised against GnRH does not induce such a response [29].

GnRH challenge in GnRH immunised mammals

Challenge of intact mammals with GnRH induces a LH and FSH response. In mammals effectively immunised against GnRH, high antibody levels prevent such a response [29]. In animals that regain fertility after a period of effective immunisation, the response appeared to be correlated with the recovery: ram lambs with testes weighing more than 100 gram showed higher LH and testosterone levels after a GnRH challenge than animals with testis weighing less than 70 gram [29].

GnRH agonist challenge in GnRH immunised mammals

Challenge of GnRH immunised animals with a GnRH agonist, which is not neutralised by the antibodies raised, causes activation of the GnRH receptor and subsequently secretion of gonadotropins. In effectively immunised animals responses to the challenge are impaired. In GnRH immunised sows for example, LH and FSH increased to levels which were approximately 25% of the levels seen in controls [29]. In castrated male ponies immunised against GnRH, the increase in LH was only 10% of the response observed in the controls after a GnRH challenge, while the FSH response was similar for immunised and control animals [29]. In contrast, challenge of passively immunised ovariectomized sheep with a GnRH agonist induced pre-treatment like LH surges [29]. The impaired LH and FSH peak values in effectively immunised animals may be due to a reduction in the pituitary gonadotropin content [29] or a reduced number of GnRH receptors in the pituitary [29]. In female mammals immunised against GnRH, pulsatile administration of GnRH agonists have been used to restore gonadotropin levels, follicle growth and ovulation. In ovariectomized ewes immunised against GnRH, pulsatile administration of a GnRH analogue resulted in elevated LH levels within 48 hours and established normal LH levels within 6 days [1]. Pulsatile administration of 100 ng GnRH agonist per hour for several days did induce follicle growth, but not ovulation. Ovulation occurred when the agonist dose was increased to 800 ng per hour [29].

Challenge of GnRH immunised mammals with gonadotropins or steroids

GnRH immunisation has been studied for the use as a male contraceptive. Therefore, the effects of testosterone supplementation in order to maintain libido after GnRH immunisation were investigated. A model for male contraception by immunisation against GnRH in combination with small testosterone implants in rats was described [2]. GnRH immunisation reduced LH and FSH to undetectable levels,

whereas a low dose of testosterone maintained libido without restoring fertility. Similar results were found in rats and rabbits [29]. It was showed that high doses testosterone were capable of restoring spermatogenesis and fertility in adult azoospermic rats without influencing serum LH and FSH levels [29]. When testosterone supplementation was started after a long period of suppressed fertility due to GnRH immunisation, effects on spermatogenesis were less pronounced, spermatogenesis could only be partially restored [29]. The role of FSH in the regulation of spermatogenesis in the absence of testosterone was also studied in GnRH immunised rats. A challenge with recombinant FSH partially restored spermatogenesis, though spermatid elongation was not restored and may require additional factors, most likely testosterone [29]. In females, effects of gonadotropin supplementation on follicle development and ovulation were studied in GnRH immunised sheep. Immunisation reduced FSH levels by 75% and as a consequence a decrease in the number of large follicles occurred. Subsequent treatment with FSH, LH and hCG caused an increase in the number of large follicles and induced ovulation, however, the ovulation rate was lower in immunised animals compared to the controls [29].

Effects of GnRH immunisation on pregnancy

The role of GnRH in early pregnancy in the rat was studied by passive immunisation using GnRH polyclonal or monoclonal antibodies. Administration of antibodies on day 4 of pregnancy caused a delay in implantation of the embryos by 5 days, but pregnancy was not terminated. This effect could be abolished by administration of GnRH or estradiol [29]. Administration of GnRH antibodies between days 7 and 10 of gestation resulted in fetal death, emphasising the importance of GnRH for the maintenance of pregnancy as indirect stimulator of progesterone production [29]. Passive immunisation of late pregnant rats at days 13 and 20 of gestation did not terminate pregnancy but resulted in reduced size of the sex organs in the pups at day 6 after birth [29]. Passive immunisation of ewes, 7 weeks before partus, impaired LH pulses in the foetuses, whereas FSH levels were either not or slightly decreased [29].

Reversibility of GnRH immunisation effects

Suppression of the reproductive axis by GnRH neutralisation is due to a sufficient amount of neutralising antibodies. Hormone levels and gonadal activity are regained when antibody titres drop below a certain level, which are insufficient to neutralise GnRH completely. Several examples demonstrating reversibility of GnRH immunisation effects have been published. Complete reversibility of the effects induced by GnRH immunisation was shown in male rats. Fertility returned to normal again between 6 to 10 months after the last vaccination, as determined by the production of litters with a normal size [16] [29]. In horses, mares with suppressed ovarian activity for up to 30 weeks after GnRH immunisation conceived and produced normal foals in the following breeding seasons when antibody titres had decreased significantly [29]. In stallions, recovery of testis function was observed and libido returned to normal again when GnRH immunisation was discontinued [8] [29]. In bull calves, testosterone and testes size increased to normal levels after 40 weeks of suppression [29], while others observed an increase in aggressive and sexual behaviour to bull-like levels when immunisations were stopped [29]. In adult dogs with reduced serum testosterone levels, antibody titres declined to pre-immune levels after a period of 28 weeks following immunisation and resulted in testosterone levels which restored to the normal range [17].

In pre- and peripubertal immunised sheep, gonadal development was suppressed for more than 90 weeks due to low gonadotropin concentrations. Surprisingly, at that time antibodies could not be detected anymore, suggesting that other factors appeared to be involved in the sustained suppression of gonadotropine levels after GnRH immunisation [29]. In these animals, secretion of GnRH in portal blood, in particularly the GnRH pulse amplitude was reduced, but GnRH concentrations in the median eminence were similar for immunised and control animals at the time they had reached the age of 3-4 year [29]. The authors suggested that this discrepancy could be caused by thickening of the blood vessel walls in the median eminence or by immune cells 'attacking' the terminal buttons of the GnRH neurons, as was previously suggested [29]. In this study in pigs, changes in the median eminence of GnRH immunised pigs were described. Damage was expressed in a lesions score, which was based on: i) a decrease in GnRH reactivity in the median eminence, ii) tissue disruption and fibrosis iii) increased accumulation of neurosecretum (Herring bodies), and iv) size of affected area of the median eminence. Although the authors interpret the observed findings as lesions, it is not unlikely that the morphological changes can be attributed to a functional atrophy. This will result in a decreased number of GnRH positive cells in the median eminence and consequently, anatomical structures involved in secretion and transport of GnRH as neurons and capillaries are atrophic. The observed presence of an increased IgG immunoreactivity in the median eminence after vaccination, which also

occurred in control animals, has not lead to influx of inflammatory cells, a hallmark of inflammation and no pathological attributes of an autoimmune process have been shown. Moreover, in a ´follow-up´ study with SPF-pigs, no evident autoimmune or inflammatory changes in the median eminence of GnRH immunised pigs were noted as compared to mock vaccinated control animals. In a separate experiment, histopathological examination of the hypothalamus of pigs effectively immunised against GnRH for 8, 16 or 26 weeks, revealed no pathological changes [29]. In general, reversibility of GnRH immunisation effects is initiated once the antibody titres are below a certain threshold level. However, in young animals sustained effects may occur, which are possibly due to impairment of hypothalamic function.

Application of GnRH vaccines

In farm animals, vaccination against GnRH could be an alternative for conventional castration methods or to prevent sexual behaviour in animals, which are normally not castrated. In pig husbandry, male piglets are surgically castrated to prevent the occurrence of boar taint in pork. An alternative for the surgical castration of young boars is urgently needed, as castration is performed without anaesthesia, which makes it a painful and animal unfriendly method. In horses, suppression of fertility and sexual behaviour can be achieved by surgical castration or hormone administration. As both approaches have drawbacks, such as the risk of surgery or daily administration of hormones and their unknown long-term effects, a suitable alternative is desired. Fattening of bulls and heifers suffers from problems related to puberty: bulls show aggressive and sexual behaviour, while heifers manifest oestrus behaviour, resulting in stress and unwanted pregnancies. Separation of males and females, surgical castration and hormone administration has been applied to overcome these problems. In male sheep and goats, several castration methods are used to prevent a reduction in growth rate, caused by sexual and aggressive behaviour. In pets and wildlife species a GnRH vaccine could serve as a contraceptive, to prevent overpopulation and unwanted pregnancies and sexual behaviour. In human health care, vaccination against GnRH could be a potential treatment for several gonadal steroid-dependent diseases, which are currently treated with GnRH agonists. It is expected that GnRH immunisation has less side effects and requires lower doses.

Pigs

Surgical castration of young male pigs is common practice in pig farming. Castration is performed to prevent the occurrence of boar taint in pork. A considerable amount of research has been carried out in order to solve the boar taint problem, nevertheless the mutilating practice of surgical castration without anaesthesia is still allowed. A non-invasive alternative such as vaccination against GnRH is a suitable option, as was demonstrated by the approval of such a vaccine in Australia (Improvac, CSL Animal Health, Victoria, Australia, recently acquired by Pfizer Animal Health).

Boar taint

Boar taint is an unpleasant odour, which can be perceived when meat of male pigs is heated. The odour is mainly caused by two compounds in the fat tissue, androstenone and skatol [19] [29]. Male pigs reach puberty between 18-21 weeks of age [29]. Pubertal development is reflected by elevated steroid production by the Leydig cells in the testis, leading to increased testicular steroid levels in the blood stream, including the pheromone androstenone [7]. Androstenone is mainly stored in the salivary glands and released in the saliva to induce receptivity of female pigs in oestrus. Due to its lipophilic character, androstenone has a tendency to accumulate in the adipose tissue in much higher concentrations than the testicular sex hormones testosterone and estradiol [7].

Skatol is a product of protein processing in the gut of the pig, in particular the processing of the amino acid tryptophan. Skatole, absorbed from the gut, is metabolised by the liver and partly accumulated in fat tissue. Its presence in fat tissue depends on the sexual maturity of the male pigs [4], as its metabolism is affected by estrogens. Increased oestrogen levels have an inhibitory effect on the metabolism of skatole by the liver and clearance from the body, resulting in increased levels of skatole in fat tissue [29]. The involvement of estrogens in skatole formation in fat tissue also explains the higher skatole levels in boars compared to sows, as oestrogen levels in boars are higher than in sows [29]. Low voluntary feed intake due to high oestrogen levels in boars, may also affect skatol concentrations as it lowers the intestinal passage, resulting in an increase in skatole formation [7]. Skatole levels in adipose tissue are also influenced by other factors, such as the energy level of the diet [7], diet composition [29], feed intake prior to slaughter [29] and housing conditions [29].

Both boar taint substances are perceived differently, the flavour of skatole is mostly related to 'manure' while androstenone is mostly perceived as 'sweat' or 'urine' [29]. For both substances androstenone and skatole threshold levels are assessed, 0.5 µg and 0.2 µg per gram fat tissue, respectively. Values

above these levels are indicative for boar taint [4].

Alternatives for surgical castration of piglets

The occurrence of boar taint makes the meat unsuitable for consumption, especially in case the meat has to be heated before consumption. The meat is also unfit for export, as most countries demand boar taint free meat. Surgical castration eliminates boar taint, but has consequences for the animal and the farmer. It is performed without anaesthesia and thus is an animal unfriendly practice which causes pain and has an impact on animal welfare [29]. In addition there is increased susceptibility to diseases [29] and it is economically undesirable, as it leads to a more inefficient feed conversion and a reduction in carcass quality [29]. Alternative procedures should preferably improve animal welfare without further costs for the farmer. Several alternatives have been mentioned, although most have disadvantages. Surgical castration with local anaesthesia is an alterative, which reduces stress and discomfort of the piglet. No difference in behaviour was observed in piglets surgically castrated with local anaesthesia as compared to intact piglets, whereas piglets castrated without anaesthesia exhibited altered behaviour, indicative of pain and discomfort [20][29]. Moreover, stress caused by castration, as evaluated by a higher heart rate and vocalisation, is reduced by local anaesthetics [31]. However, surgical castration with the use of anaesthesia requires veterinary assistance, which makes this approach costly and practically impossible.

Detection of boar taint at slaughter would be preferable as it allows the production of intact male pigs. In Denmark, slaughter line detection of boar taint was applied in the nineties, but only skatole levels were determined, which appeared to be insufficient to detect all tainted carcasses. Successful detection requires analysis of both substances, androstenone and skatole. However, the lack of a high throughput system for measuring androstenone hampers application of this method. The use of an electronic nose, which measures both substances at one occasion, has been successfully applied in an experimental setting [29]. All tainted carcasses were identified, however 16% of the carcasses were classified false positive. Although this alternative may look promising, it does not solve the problem of the high incidence of boar taint, which is about 60% in Europe [29].

Slaughtering of pigs at a lower body weight certainly will reduce the incidence of tainted carcasses, but additional slaughter line detection is still necessary to identify tainted carcasses. At a body weight of 80-90 kg still more than 5% of the carcasses exhibits boar taint [3] [29]. Moreover, lighter carcasses are less profitable for pig farmers and may affect pork export.

Another possibility to control the occurrence of boar taint is by selection for low concentration of boar taint compounds. The use of sires and dams selected for low fat androstenone concentrations, lowered androstenone concentrations in the offspring as compared to pigs selected for high androstenone levels [29]. However, no significant differences with the control group were seen. Moreover, selection for low androstenone concentrations negatively affected growth rate, which can be explained by the fact that selection against androstenone is associated with late maturity.

Skatole levels in intact male pigs can be influenced by selection for growth rate on diets with different protein levels. Pigs selected for growth rate on a high protein diet had significantly lower skatole levels than pigs selected for growth rate on a low protein diet [29]. Although, the different protein sources used for both diets may also contribute to the observed differences in skatole levels.

Sperm sexing has proven to be effective in producing litters of the desired sex [29]. However, it is unlikely that this technique will be implemented in pig husbandry in order to produce predominantly female piglets. Conventional artificial insemination requires about 2 to 3 billion spermatozoa, which is far above the capability of sorted sperm production [29].

Immunisation against GnRH

To circumvent the disadvantages of surgical castration, immunisation against GnRH seems a feasible option. Disruption of the hypothalamic-pituitary-gonadal axis by neutralisation of GnRH, leads to a reduction of LH and FSH production and secretion, thereby inhibiting maturation of the testes in growing animals as well as the synthesis of testicular steroids among which androstenone, testosterone and estrogens. This approach makes surgical castration redundant as concentrations of the boar taint causing compounds, androstenone and skatole, are reduced to levels similar to surgical castrated pigs [29]. Several studies have been performed, focusing on the occurrence of boar taint in GnRH immunised boars. A vaccine was used consisting of GnRH conjugated to HSA with CFA or MDP as the adjuvant. Immunisation at 12, 16 and 18 weeks of age resulted in undetectable LH and testosterone levels and reduced weight of testes and accessory sex glands [29]. Moreover, the incidence of boar taint was reduced. Furthermore, pituitary and Leydig cell function were studied [29]. Undetectable serum LH and testosterone levels were accompanied by a reduction in pituitary LH content and testicular LH receptor content. However, plasma FSH concentrations and pituitary FSH

content were not affected. In a subsequent study, the morphology of the testes of GnRH immunised boars was examined [29]. Changes were seen in the seminiferous tubules: diameter and epithelium height were reduced, spermatids were absent and Sertoli cells structure was affected. Due to the depletion of LH, size, number and activity of the Leydig cells was reduced. Effects on sperm counts were studied in 11-month-old boars by [29]. Five immunisations with a GnRH-BSA conjugate in CFA reduced LH, testosterone and testes volume. In these mature boars, testes weight was reduced by 66% and the number of sperm cells was reduced by more than 75%.

Growth performance of GnRH immunised boars was studied [5]. Immunisation at 29 and 89 kg body weight with a GnRH conjugate in mineral oil and in an aqueous solution with saponin as an adjuvant, respectively, resulted in highly variable antibody titres, ranging from undetectable to substantial levels. Mean testis weight was reduced as were plasma testosterone concentrations and adipose androstenone levels, while growth performance, i.e. average daily weight gain and feed efficiency, of the immunised boars was similar to that of intact boars and improved compared to surgical castrates.

Our group demonstrated that testis size or testis weight could serve as a parameter for absence of boar taint in immunised pigs [23]. Pigs immunised against GnRH at 10 and 18 weeks of age and slaughtered at 26 weeks of age, always had androstenone levels below 0.5 µg/g when testis weight was less than 150 g or testis size measured prior to slaughter was less than 9 cm. In Chinese cross-bred pigs, a modified GnRH-tandem-dimer molecule conjugated to OVA and emulsified in Specol adjuvant [29], decreased serum testosterone and adipose androstenone levels, and caused a reduction in testis weight in 35 out of 39 treated male pigs [29]. Effects of this vaccine on growth performance were studied in pigs fed diets with a high or a low energy content. For both diets, feed intake of the immunised boars was similar to the surgically castrated littermates, while the energy conversion ratio was in between those of intact boars and surgical castrates. This resulted in a growth rate of the immunised boars which tended to be higher than intact boars and surgical castrates [32].

A recombinant fusion protein containing leukotoxin and several copies of a GnRH-tandem molecule in a commercially acceptable oil-in-water adjuvant was also evaluated as a boar taint vaccine [29]. In this study, boars received the first vaccination at 3 weeks of age and a booster when they reached 100 kg bodyweight. This vaccination lowered serum testosterone and fat androstenone concentrations to castrate levels in all 11 treated pigs. However, no follow-up studies in pigs have been published using this vaccine.

The first commercially available boar taint vaccine, Improvac (CSL Animal Health, Victoria, Australia), was approved in 1998 for prevention of boar taint in male pigs in Australia. Castration is not common in Australian, however, the export of pork to Japan required the production of heavier animals. In order to avoid a high incidence of boar taint carcasses, a vaccine against GnRH was introduced. The vaccine comprises a GnRH peptide conjugated to a carrier protein, mixed with non-oil based adjuvant. More details about the vaccine were not published. Two doses of 2 ml vaccine are advised to be administered 8 and 4 weeks before slaughter to efficiently reduce boar taint. The vaccine should be administered using special equipment, the Improvac vaccinator gun, thereby injecting the vaccine in the skin.

The effects of Improvac in boars slaughtered at 23 or 26 weeks of age was studied [9]. Testosterone levels were reduced after the second vaccination, however, at time of slaughter still 8% of the immunised boars had testosterone levels above 2 ng/ml, which is substantially higher than the levels in castrated pigs (0.3 ng/ml). Mean testis weight of Improvac-treated boars was reduced by 50% as compared to intact controls. The authors stated that in pigs treated with Improvac, testes weight should be less than 350 and 400 gram for pigs slaughtered at 23 and 26 weeks of age, respectively, to ensure boar taint free carcasses. However, based on these threshold levels it is not possible to discriminate between treated and untreated boars, as a substantial number of the untreated boars had testes weights below these values. Growth performance of the Improvac-treated boars was altered after the second vaccination. Treated boars grew faster than intact boars and had better feed conversion efficiency and leaner carcasses than castrates. Moreover, Improvac-treated boars were less aggressive, only 4% exhibited wounds around the head and shoulders due to fighting compared to 26% of the intact boars. Tissue reactions due to injection of the vaccine were observed in 15 to 20% of injection sites. Androstenone and skatole levels of Improvac treated boars were significantly reduced as compared to intact boars and were similar to castrated boars. However, 6% of the Improvac-treated boars that were slaughtered at 26 weeks of age had androstenone levels exceeding the threshold of 0.5 µg/g back fat and 2% had skatole levels above 0.2 µg/g back fat. In a field study including 319 vaccinated boars, 2.5% and 2% of the vaccinated pigs had values above the threshold for androstenone and skatole, respectively [29].

Horses

Alternative for castration

In horses, GnRH immunisation can be used as an alternative for surgical castration of stallions. Surgical castration, although commonly applied, is not without risks as it causes complications in more than 5% of the treated stallions [29]. Castration of cryptorchid stallions even has a higher risk for complications [29]. Therefore, a non-surgical castration method without the risk for complications is preferable. Several hormone treatments have been applied in order to reduce libido and aggressive behaviour, but efficacy of these treatments is limited, costs are high and frequent administration is required [29]. An advantage of GnRH immunisation is the reversibility of the induced effects. When GnRH immunisation is not repeated, a recovery of the testis function can be observed and libido will return to normal again [8]. Such a reversible ´castration´ method can save the reproductive capacity of exclusive stallions that are involved in all kinds of sports and normally will be surgically castrated because of their undesired libido and aggressive behaviour. As in stallions, the most important reason for GnRH immunisation studies in mares is prevention of undesirable sexual behaviour. Behavioural changes may interfere with performance in sporting competition and with handling of the mares. In addition, some mares exhibit continues oestrus behaviour, due to hormonal changes.

GnRH immunisation may be considered an alternative for castration for medical reasons, e.g. for treatment of persisting equine arteritis virus (EAV) infections in stallions. After the acute phase of the disease, infection of stallions frequently leads to a carrier state in which virus is continually shed with the semen. The presence of testosterone is essential for the persistence of the virus in the stallion [29]. A temporarily intervention in testosterone production, as established by GnRH immunisation, could be sufficient to clear the infection. Infections in mares are usually mild and transient, but may lead to termination of pregnancy.

Immunisation against GnRH

The first GnRH immunisation study in horses was performed successfully in a cryptorchid stallion; testosterone concentration was reduced and the stallion behaved docile. In this study, CFA was used as an adjuvant which is not allowed be used in practice, as it may induce severe reactions at the site of injection. Local reactions at the injection site were also observed in colts immunised with a mineral oil-based GnRH-vaccine [29]. In this study all treated stallions showed depressed testosterone levels and testis function appeared to be affected. However, the occurrence of adverse reactions after vaccination is not acceptable for a horse owner. Therefore the vaccine should preferably contain an adjuvant which does not induce injection site reactions. A GnRH vaccine containing a water-soluble adjuvant in young stallions was tested [8]. Two vaccinations caused a decrease in testosterone concentrations and absence of libido, which lasted for 3 to 6 months. Unfortunately, this vaccine has not been applied in sexually mature stallions, which most likely will be the most important target group. Three adult stallions were treated with a GnRH vaccine comprising Equimune adjuvant [29]. This vaccine failed to induce high antibody titres in one of the three stallions, despite the fact that five immunisations were given. Moreover, local tissue reactions were observed in all treated stallions.

In mares, immunisation against GnRH suppressed ovarian activity [29]. In these studies CFA was used as an adjuvant, whereas the use of Equimune adjuvant resulted in a partially effective vaccination [29]. A water-soluble vaccine was shown to suppress fertility for 20 weeks in 4 young mares [29]. Recently, CSL Animal Health (Victoria, Australia) launched a GnRH vaccine, called Equity, for oestrus control and prevention of oestrus related behaviour in mares. Vaccination results in suppression of ovarian activity subsequently leading to reduced oestrus behaviour, however, according to the medication guide results are variable: not all mares respond (http://www.pfizeranimalhealth.com.au/).

Cattle

Bulls grow more efficiently than castrated bulls and produce leaner carcasses. However, to prevent aggressive and sexual behaviour, most bulls fattened in pasture based systems and feedlots are castrated. Several castration methods can be applied, which all cause stress and pain [29]. In beef cattle, pregnancy and oestrus behaviour impairs growth rate, whilst oestrus and pregnancy in dairy cattle to be culled, cause a reduction in milk production. Immunisation against GnRH has been studied in order to suppress fertility and sexual behaviour in cattle. It was shown that GnRH immunisation reduced testosterone levels in bull calves [29]. Further studies showed docile behaviour of the treated bull calves, improvement of growth performance as compared to castrated bulls and reversibility with respect to hormone levels [29]. In beef heifers, GnRH immunisation was effective in preventing pregnancy [29]. In these studies, multiple vaccinations were given with CFA as the adjuvant.

A single immunisation protocol was tested [29]. Five mg GnRH-KLH conjugate in CFA reduced testes weight in 12-month-old bulls, however, at slaughter at 18 months of age, the vaccine appeared not to be effective anymore: testosterone levels were again at the same level as those of the untreated bulls. Similar results were found when a single immunisation was given at a younger age, i.e. at 4 or 7 months of age [29]. Two immunisations, the first one at 1, 4 or 6 months of age and a booster at 12 months, did result in reduced testosterone levels at slaughter [29]. In these bulls, growth performance was in between that of intact and castrated bulls, while aggressive behaviour was reduced to castrate-like levels [29]. In beef heifers, the use of GnRH immunisation and daily oral hormone administration (melengestrol acetate, MGA) was compared in order to prevent oestrus behaviour and pregnancy. Both, MGA treated heifers and heifers treated with the GnRH-KLH conjugate in CFA were not cyclic. Weight gain and feed efficiency were not different between intact, MGA treated and GnRH immunised heifers, indicating that GnRH immunisation can be used as an alternative for MGA administration.

Vaxstrate, a GnRH vaccine, which consists of a GnRH decapeptide conjugated to OVA in a DEAE Dextran/mineral oil mixture, was approved in Australia to prevent oestrus behaviour and pregnancy in cattle [14]. Five ml emulsion must be administered subcutaneously, followed by a booster immunisation 4 to 16 weeks later. Although pregnancy rates were low in case of a vaccination interval of 4 weeks, a 16 week vaccination interval resulted in pregnancy rates of 20%, while pregnancy rates in the control group were 63%. The effects of Vaxstrate on growth performance were studied [29]. Growth performance and carcass value of Vaxstrate treated heifers appeared to be in between those of intact and surgically spayed heifers. However, the costs of spaying were less than the costs for GnRH vaccination, 4 vaccinations at 5.5 Australian dollars each, making GnRH immunisation a less attractive alternative. Bulls were immunised with Vaxstrate at 2, 4 or 7.5 months of age and boostered 1 or 2 times several weeks later [29]. Two or 3 immunisations appeared not to be sufficient to suppress testosterone levels and sexual behaviour until slaughter at 18 months of age. A single immunisation with a Vaxstrate-like vaccine containing a GnRH-HSA conjugate in a DEAE Dextran/mineral oil mixture with bacteria incorporated was evaluated [29]. Immunisation of 8-month-old heifers delayed puberty by 3.5 month, but also reduced growth performance. When the conjugate was administered in DEAE Dextran without mineral oil, 2 immunisations were required to reduce testosterone for a similar period in bull calves [29]. Though antibody responses were variable in these bulls, aggressive and sexual behaviour was reduced [29].

A mixture of two GnRH fusion proteins, based on ovalbumin and thioredoxine, administered in CFA suppressed LH and testosterone concentrations in bulls after the second vaccination, while in heifers, 3 vaccinations with the antigens in a water-in-oil emulsion containing bacteria as an immunostimulant, resulted in a cessation of the oestrus cycle in all 39 treated animals [29]. Immunisation with a recombinant fusion protein consisting of leukotoxin and several copies of GnRH was studied using a commercially acceptable adjuvant [29]. Due to variable responses, mean testosterone levels were only slightly reduced. The authors concluded that more research is needed to reduce variability in the response to the vaccination and to optimise the timing of vaccination.

Sheep and goats

In two early GnRH immunisation studies in ram lambs, a GnRH-BSA conjugate in CFA was used. Ram lambs were immunised 3 times, 6 weeks apart, which resulted in a significant reduction in testis size from 13 weeks onwards, while sperm motility was affected in week 24 to 26 [29]. In the second study, young ram lambs were immunised twice, resulting in castrate-like testosterone levels and 80% reduction in testis weight at 6 weeks after the second vaccination. Growth performance and carcass traits were equally affected by GnRH immunisation and surgical castration [29]. A single vaccination protocol was evaluated in Chinese Tanyang ram lambs immunised at 3 months of age with a GnRH-BSA conjugate in CFA. In these animals serum testosterone was suppressed and testis growth was affected for 4 months, while the onset of sexual behaviour was delayed [29]. Undetectable testosterone levels were also reported in ram lambs immunised with a GnRH-KLH conjugate in CFA at an initial body weight of 33 kg. Sexual behaviour of the immunised lambs was similar to castrates, as were growth performance and carcass characteristics. Immunisation with the conjugate in ISA, also reduced testosterone levels, but levels remained higher than in CFA treated animals.

Two immunisations with the ´boar taint vaccine´ Improvac, given 3 weeks apart, suppressed testosterone in ram lambs [29]. However, 8 out of 10 treated animals showed an increase in testosterone between 3 and 7 months after immunisation.

In adult male goats the effects of the 'cattle vaccine' Vaxstrate was studied [29]. Two subcutaneous injections given either 2 or 4 weeks apart resulted in reduced levels of LH, FSH and testosterone, which decreased more rapidly in the animals that received fortnightly injections. Semen characteristics

were affected: sperm motility was affected at day 112, 175 and 329, sperm counts were reduced at day 175 and 329, while ejaculate volume was only reduced at day 329 after the first immunisation. Buck odour score, which was only determined until 8 weeks after the first injection, was reduced between 6 and 8 weeks in the animals vaccinated 2 weeks apart. Aggressive behaviour was reduced in both vaccinated groups compared to intact male goats.

Pets

Pet overpopulation is a big problem in many countries. Stray dogs and cats are causing damage to property, spreading diseases and attacking the public, livestock and wildlife. In the US, every year about 20 million dogs and cats are euthanised to decrease the population of unwanted pets [29]. In the Netherlands, five million cats are kept by private owners. Although methods to prevent pregnancies are used, the number of unwanted cats that are discarded is still increasing. Every year ten thousands of cats are brought to shelters in the Netherlands. Neutering is the most effective way to prevent pregnancies in pets. However, costs are high, it is laborious, anaesthesia is required, post-surgical infections may occur and it is not reversible. Alternatives have been developed for this purpose. Several progestagens are on the market, which are effective in suppression of oestrus in female cats and dogs by inhibition of gonadotropin release [29]. However, frequent administration is necessary and undesirable side effects may occur like uterine diseases, diabetes, mammary tumours, increased appetite and weight gain. The effects of GnRH immunisation on fertility and sexual behaviour in pets have only been evaluated in a limited number of experiments. In dogs, most studies have been performed in males, presumably due to the long oestrus interval in females.

In the first study in dogs [29], sexually mature males were immunised subcutaneously with 200 µg GnRH conjugate in CFA at 3 monthly intervals. Two out of 5 dogs responded with reduced LH and testosterone levels while testes weights were reduced by 70%. Others showed a response in 2 out of 2 young beagles which were intradermally treated with a GnRH-tuberculin protein conjugate. An extensive study was performed with GnRH conjugated to TT in Pluronic block polymer L121/MDP analog adjuvant [17]. Five sexually mature male dogs were immunised at 0, 2 and 4 weeks. After the second immunisation serum testosterone concentrations were reduced to castrate levels and maintained low for 28 weeks. At that time antibody titres had declined to pre-immune levels, resulting in a rise in testosterone levels.

A single immunisation protocol using GnRH-BSA in Titermax adjuvant or Alum, did cause a decrease in testes size, especially in the Titermax group, but testosterone levels were only reduced for 2 weeks [29]. The lack of efficacy using Alum in dogs was also reported [29], when a GnRH-TT conjugate was used and also when a synthetic GnRH antigen comprising the GnRH decapeptide and T cell epitopes was used [11]. However, the latter antigen in an oil-in-water emulsion (Emulsigen combined with DDA) was effective. Testosterone was reduced to castrate levels after 3 intramuscular injections until the end of the study, 14 weeks after the third immunisation. In another study, 10 cyclic female dogs were immunised 4 or 5 times within 26 weeks with a GnRH-TT conjugate in CFA, MDP/Alum or LPS/Alum[29]. Oestrus was blocked in all dogs, except 1 of the 4 animals treated with the MDP-adjuvanted vaccine. It was demonstrated that intramuscular administration of a GnRH-KLH conjugate in squalane combined with MDP resulted in a higher GnRH antibody titre than subcutaneous administration [29]. The latter route of administration resulted in oestrus in all bitches, while in intramuscularly treated animals oestrus was prevented in 5 out of 8 dogs.

Differences between species in their response to GnRH immunisation have been reported [17]; the vaccine studied was fully effective in dogs but appeared to be only partially effective in male cats. A study in which 10-month-old male cats were immunised 4 times during a period of 12 weeks with a GnRH-OVA conjugate in DEAE-Dextran was reported [29]. At week 20, testosterone levels and testis size were reduced and spermatogenesis was completely suppressed. In a recent study, a single immunisation with a GnRH-KLH conjugate in a novel oil-based adjuvant called AdjuVac^{TM}, containing Mycobacterium avium, reduced testosterone levels to zero for at least 4 months in 6 out of 9 one-year-old male cats [29]. Two-month-old male and female cats were immunised by subcutaneous injections of 0.25 ml vaccine given at 2 occasions 4 weeks apart [24]. The antigen, a recombinant leukotoxin-GnRH fusion protein was administered in Emulsigen combined with DDA. Immunisation suppressed testosterone concentrations until day 300 of the study in 4 out of 4 male cats, while all 10 immunised females were in anoestrus as determined by low progesterone levels until day 600 of the study. After immunisation, tissue reactions were observed at the injection site, but the majority of the reactions had disappeared by day 28.

Wildlife species

In wildlife species, GnRH vaccination may offer a tool for population size control. In white-tailed deer

immunisation with a GnRH-KLH conjugate in CFA led to reduced oestrus behaviour, while fawning rates were reduced by 88%. Infertility lasted for up to two years [22]. Another study in female white-tailed deer showed that a GnRH-OVA conjugate in DEAE Dextran did not disrupt oestrus cycle [29]. The authors suggested that the lack of efficacy may be due to the applied carrier protein or the timing of immunisation with respect to the phase of the reproductive season. In male red deer, immunisation against GnRH prevented a reduction in feed intake and growth during the rut period and resulted in heavier animals as compared to untreated controls, as a result of the temporary suppression of testosterone levels [29]. Furthermore, males successfully immunised, casted their antlers prematurely during autumn instead of during spring [29].

The effects of GnRH immunisation on social behaviour were studied in brushtail possums, which is a host species of bovine tuberculosis in Australia [29]. Social hierarchies remained unchanged after vaccination of dominant females. We showed that an aqueous GnRH vaccine suppressed testosterone concentrations in young zoo-animals (blackbucks, springbok antelopes and goat bucks), while hormone levels were not affected when an adult blackbuck and springbok antelope were vaccinated [29]. In sexually mature elephants, however, the vaccine caused a reduction in gonadal steroid production as determined by reduced faecal epiandrosterone concentrations. In addition, bulls did not show aggressive behaviour anymore [29]. A single-injection programme with the GnRH vaccine GonaCon™, has been developed by the National Wildlife Research Centre in the USA to control population size of wildlife species [29]. The vaccine, which contains a GnRH peptide conjugated to KLH combined with AdjuVac™ adjuvant is in the process of approval by the FDA.

Humans

Male contraceptive

In the past, several research groups focussed on GnRH immunisation as a means for non-surgical male contraception. In order to maintain libido after GnRH immunisation, exogenous testosterone had to be administered. A model for male contraception was described using GnRH immunisation against GnRH in combination with small testosterone implants in rats [2]. Low doses exogenous testosterone maintained libido in GnRH-immunised azoospermic rats. Low doses of testosterone maintained copulation behaviour and ejaculation in rats and rabbits, but the animals were not fertile. It was shown that high doses testosterone are capable of restoring spermatogenesis qualitatively in adult azoospermic rats while serum LH and FSH remained undetectable [29]. Moreover, animals were fertile as determined by the production of litters of normal size. The vaccine used in the study [29] was evaluated in a Phase 1 Safety study in male volunteers. The immunisations were well tolerated, but responses were variable; some men responded immediately to the immunisation, while others showed a late response or did not respond at all (www.popcouncil.org). Because of the inconsistent response, further development of this vaccine was stopped.

GnRH immunisation combined with testosterone supplementation resulting in infertility with maintenance of libido, could be a suitable contraceptive in men. However, besides the inconsistent responses, several factors have hampered the application of GnRH immunisation as a male contraceptive. For instance, the availability of several appropriate contraceptive methods and the expected difficulties getting such a vaccine approved for healthy humans, considering the side effects of an adjuvanted vaccine which has to be administered on a regular basis.

Anticancer therapy

As the GnRH vaccine effectively reduces gonadal steroid levels, it could be a promising tool to treat gonadal steroid-dependent diseases such as prostate cancer, breast cancer and endometriosis. Here, we focus on prostate cancer.

Prostate cancer

Prostate cancer is the second most common type of cancer in men. Worldwide more than 650.000 new cases are diagnosed every year of which about 190.000 in the US and 190.000 in the EU including 6000 in the Netherlands. The incidence rates are severely influenced by the availability of testing in a population. For instance, the incidence in the USA is twice as high as in most European countries, due to PSA testing in the USA. In Europe, an estimated 30% of the diagnosed men die from the disease (www.afud.org).

Treatment of prostate cancer depends on the age of the patient and the stage and the growth rate of the tumour. In older men with slow growing tumours no treatment is advised as side effects of the treatment may outweigh the benefits. Prostatectomy is the most common treatment for early stage prostate cancer. It is a successful therapy, but has a high risk for impotence and urine incontinence. Radiation therapy can be applied in early stage tumours as the primary therapy, in cases surgery can

not be performed or after surgery to prevent recurrence of the cancer. In metastatic prostate cancer, which up to now is not curable, radiation therapy relieves the pain. Hormone therapy is mainly applied when the cancer has metastasised or to prevent recurrence after prostatectomy or radiotherapy. It blocks the release of male steroids, which are necessary for prostate tumour cell growth. Hormone therapy includes: (i) surgical castration, (ii) GnRH agonist treatment, in order to prevent the testes from producing androgens by overstimulation of the GnRH receptor, which results in a reduction in LH and FSH release by the pituitary and (iii) anti-androgens treatment, which blocks the action of androgens by occupying the androgen receptor in the tumour cells. Although commonly used, these therapies all have side effects. Surgical castration and GnRH agonists are causing impotence and loss of libido. GnRH agonist treatment also causes a so-called flare-up reaction. The agonist initially enhances testosterone production by overstimulation of the GnRH receptors, resulting in enhanced tumour growth for some weeks. Gradually, however, the treatment causes testosterone levels to drop to very low levels. For this reason GnRH agonist treatment can not be used as mono therapy in patients with spinal cord metastasis as nerve compression may occur. Anti-androgens, which are given to avoid the flare-up reaction or as combination therapy with GnRH agonists, are causing nausea, vomiting and diarrhoea, whereas long term treatment may cause liver problems as well as gastrointestinal side effects. GnRH vaccination could be an alternative for hormone therapy, as it reduces testosterone to undetectable levels, without inducing a flare-up. This also implicates that GnRH vaccination can be applied in patients with spinal cord metastasis without the use of anti-androgens to counteract the flare-up response. Other advantages of vaccination against GnRH as compared to the commonly used GnRH agonist therapy, could be the lower frequency of administration and lower costs of the treatment.

Clinical trails

Several GnRH vaccines have been developed for the treatment of prostate cancer. The group of Talwar used a vaccine comprising a modified GnRH decapeptide with a D-Lysine at position 6 linked to diphtheria toxoid (DT). This vaccine reduced testosterone concentrations to castrate levels and caused atrophy of the reproductive organs in rats and monkeys [29]. The vaccine was also studied in rats bearing androgen-dependent prostatic tumours (R3327-PAP) [29]. These so-called Dunning tumour cells are implanted under the skin and develop into a measurable tumour. After three monthly immunisations tumour growth was suppressed compared to untreated controls. Surprisingly, tumour growth was also suppressed in rats implanted with androgen-independent Dunning tumour cells (R3327-AT2.1). The authors suggest that a local GnRH-loop exist in the prostate, which is affected by GnRH neutralising antibodies, causing a reduction in tumour growth even in testosterone-independent tumours.

Acute and chronic toxicology studies with this vaccine have been performed in rats and monkeys. Three intramuscular injections given at monthly intervals, showed no detrimental effects, whereas testosterone levels declined in all treated animals [13]. Clinical trials in patients with advanced prostate cancer revealed that in contrast to rodents and monkeys, high antibody titres were obtained in some, but not all treated patients. A reduction in prostatic size was observed in 3 out of 6 patients treated with 400 μg conjugate in Alum, whereas 1 out of 6 patients treated with 200 μg conjugate responded [28]. The vaccine was also evaluated in postmenopausal women with elevated LH and FSH levels due to severe hypoestrogenism [13]. After 2 injections with 300 μg GnRH equivalent of the conjugate in Alum, LH and FSH levels were decreased for 4 months in all 3 patients [29]. Recently, a recombinant GnRH vaccine was developed [29]. Reason for this change in type of antigen, were the high costs for the synthesis of their GnRH-DT conjugate, possible difficulties with the reproducibility when manufactured on a large scale, and the occurrence of carrier-induced immune suppression in some treated subjects due to the use of DT [29]. The recombinant product consists of 6 GnRH repeats and five different promiscuous T-cell epitopes. Multiple vaccination with CFA as the adjuvant resulted in significantly reduced testes and accessory sex gland in all treated rats.

The GnRH antigen developed by the company Aphton (Woodland, USA) comprises the GnRH molecule extended with a linker peptide of 6 amino acids conjugated to DT. In their first study, 12 men with advanced prostate cancer were vaccinated with 30 or 100 μg conjugate in a water-in-oil emulsion [25]. Four patients, 2 of each dose group, responded with a reduction in testosterone and PSA levels for 70 to 250 days. Three and 15 μg doses were evaluated in order to determine the minimal effective dose [29]. Suppression of testosterone to castrate levels was detected in 2 out of 6 patients treated with 15 μg antigen, whereas none of the patients treated with 3 μg responded. Antibodies raised against the Aphton GnRH vaccine have also been tested in an animal model for human breast cancer. Passive immunisation with purified antibodies raised in rabbits inhibited growth of oestrogen-sensitive

MCF7 human breast cancer tumours in nude mice [29].

The Population Council in New York, who first focussed on an antifertility vaccine for men, studied their vaccine in rats bearing androgen-sensitive prostate tumours [29]. The vaccine, which contains a native GnRH molecule with glutamine at position 1 for conjugation to TT, was evaluated in combination with GnRH antagonist treatment. It appeared that tumour size in the animals receiving the combined GnRH antagonist plus GnRH vaccine treatment was significantly lower compared to animals receiving a single treatment. Clinical trials in prostate cancer patients have not been performed so far.

The company Biostar, nowadays known as MetaMorphix Canada (Saskatoon, Canada), developed a GnRH vaccine comprising a recombinant fusion protein produced in E. Coli bacteria: several copies of a GnRH-tandem molecule were fused to the terminal ends of leukotoxin. This vaccine has shown full efficacy in young pigs and cats [24] [29], while antibody responses were variable in heifers [29]. For application in prostate cancer patients, the vaccine called Norelin^{TM}, was out-licensed to York Medical BioSciences (Mississauga, Canada). In 2001 clinical studies indicated that the vaccine with an aluminium salt-based adjuvant was safe to be used in humans, however it was not immunogenic enough to raise a sufficiently strong immune response. In 2003, a second clinic trial was initiated. This vaccine was well tolerated with ´no major adverse events´. Immune responses could be determined in 60% of the patients (www.ymbiosciences.com).

United Biomedical (UBI, Hauppauge, USA) developed a complete synthetic vaccine comprising the GnRH decapeptide and several promiscuous T-cell epitopes. Immunogenicity of the GnRH-T cell epitope constructs was enhanced by addition of a domain from Yersinia invasin protein [11]. Although the single constructs were not completely effective in rats, mixtures of constructs caused serum testosterone to drop to very low levels, whereas testes weights were less than 25% of the controls. The antigens in a water-in-oil formulation and oil-in-water formulation were effective in baboons and dogs, respectively. Furthermore, androgen-responsive Dunning prostate tumour cell growth was reduced as a result of vaccination. Three initial vaccinations followed by two boosters several month later, blocked tumour growth for more than 60 weeks. Phase 1 clinical trials have been performed and follow-up trials were planned for 1999, however, results have not been published so far. In a recent press release UBI announced trials in prostate cancer patients in China (www.unitedbiomedical.com).

Proterics (formerly known as Proteus) developed a GnRH vaccine ´Prolog´, which was out-licensed to ML Laboratories. They completed phase II clinical studies in 2000, but at present no results have been published (www.mllabs.co.uk). The vaccine containing the GnRH decapeptide with an additional glycine and cysteine has been tested with variable results in several animal species [29].

Pepscan's improved vaccine

Immunisation against GnRH as a method to reduce gonadal steroid secretion has been studied for many purposes. In farm animals it has been used to improve growth performance, meat quality and handling of the animals and to prevent pregnancies and undesired sexual behaviour [29]. In humans, immunisation against GnRH has potential for treatment of endocrine disorders [13] and treatment of gonadal steroid-dependent tumour growth [25] [29]. So far, implementation of GnRH vaccines in practice has been hampered mainly because of the low efficacy of the vaccines, i.e. the inability to raise a sufficient immune response in order to block gonadal steroid production in each vaccinated individual. Various attempts have been made to solve these problems, like modification of the antigen, the use of more effective carrier molecules and more immunostimulatory adjuvants. Since the choice of adjuvants and carrier molecules is rather limited, we have started the present study. Methods have been developed to enhance the immunogenicity of the antigen itself. It was shown that the immunogenicity of the native GnRH molecule can be increased by enlargement of the GnRH peptide to a tandem molecule, whereas it becomes even more immunogenic when the tandem peptide is dimerised and non-native amino acids are introduced. Moreover, the specificity of the antibodies can be further modified by amino acid replacements within the antigen itself. The optimised antigen appeared to be fully effective in combination with different carrier proteins and adjuvants in pigs. GnRH vaccination in pigs improved growth performance as compared to surgical castrated pigs. In horses, full efficacy was obtained with the optimised antigen in a well tolerated adjuvant. The vaccine is not toxic. A chronic toxicity study performed in pigs, in order to be able to start a Phase 1 clinical trial in prostate cancer patients, showed that no significant abnormalities were induced after short-term and long-term exposure to high levels of GnRH neutralising antibodies.

Increased immunogenicity of GnRH-tandem peptides

A highly efficient GnRH vaccine was developed by enlarging the GnRH molecule to a GnRH-tandem construct [21]. It was showed that the GnRH-tandem and GnRH-tandem-dimer peptides are

immunogenic by itself and immunosterilised, i.e. block testes growth and function in male piglets effectively (immunosterilisation was previously called immunocastration, however as the testes are not removed but only temporary inactivated, immunosterilisation is a more appropriate name)[29]. Immunogenicity was further increased by conjugation of the peptides to a carrier protein. Conjugation of a GnRH-tandem-dimer peptide, in which a glycine at position 6 was substituted by a D-lysine, to ovalbumin (OVA) resulted in a highly effective antigen (G6k-GnRH-tandem-dimer) which appeared to be slightly more effective than the originally prepared GnRH-tandem peptide (Figure 4). The G6k-GnRH-tandem dimer peptide conjugated to OVA and emulsified in Specol adjuvant showed the highest efficacy with 9 out of 9 pigs responding, as determined by very low testes weights. A dose response study with this vaccine revealed that even with peptide doses as low as 5 µg almost complete effectivity could be obtained. This vaccine has been tested extensively in male piglets. Vaccination with peptide doses ranging between 50-150 µg emulsified in Specol adjuvant substantially inhibited testis growth in 98% out of a total of 200 vaccinated pigs (unpublished results). In Chinese cross-bred pigs, the vaccine also caused a reduction in testis weight in 35 out of 39 treated male pigs, whereas 10 out of 12 vaccinated female pigs showed decreased LH and inhibine levels and a reduction in ovarian weights [29].

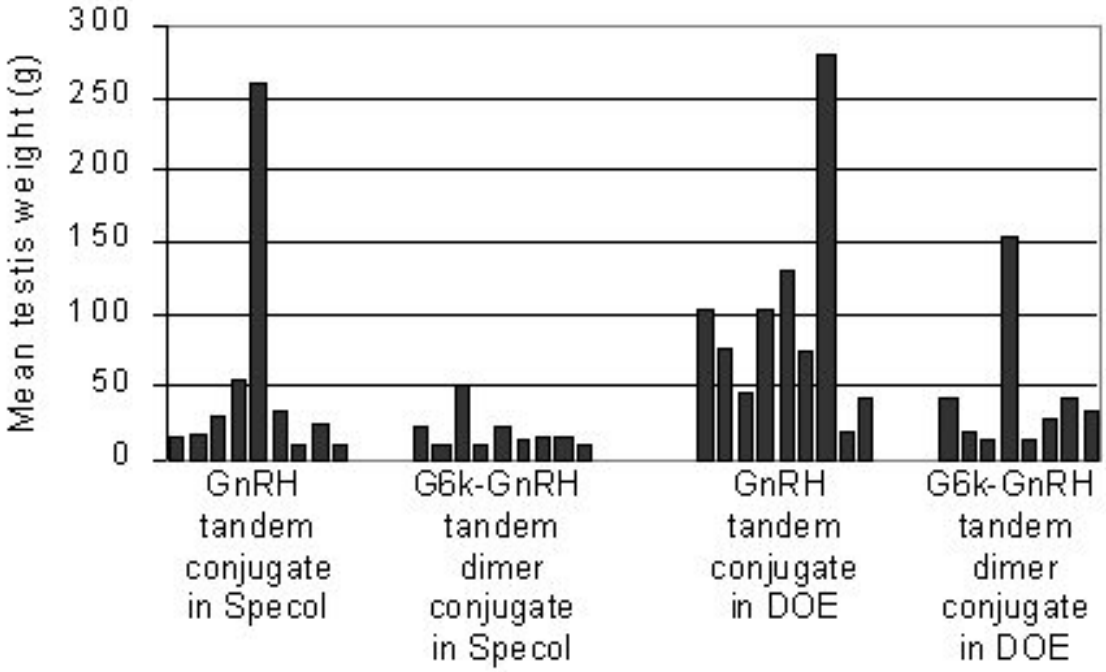

Figure 4: Mean testes weight of pigs following immunisation with GnRH-tandem-OVA conjugate or G6k-GnRH-tandem-dimer-OVA conjugate in Specol adjuvant or Double Oil Emulsion (DOE). Testis weight of untreated controls ranged between 200 and 300 g. Responders are defined as pigs with a testis weight less than 150 g [23].

Molecular alterations: effects on efficacy and specificity

The role of the individual amino acids of the G6k-GnRH-tandem-dimer was investigated [29]. This was done by replacing each amino acid of the decapeptide one at the time by alanine. These constructs were evaluated for their ability to establish immunosterilisation in male piglets. It was shown that 4 out of 9 amino acids (amino acids at position pE1, S4, R8 and G10 of the decapeptide of the G6k-GnRH-tandem-dimer) can be individually replaced by alanine without affecting the efficacy of immunosterilisation. Replacement of amino acids in 3 other positions (position Y5, L7 and P9) resulted in a reduction of the efficacy, as 5, 6 and 6 out of 7 piglets were immunosterilised, respectively. Replacements at position H2 and W3 did not lead to immunosterilisation. These results indicate that several amino acids can be replaced in order to make the peptide more ´foreign´, without affecting the ability to induce the formation of GnRH neutralising antibodies.

In the past few years, the presence of several non-mammalian GnRH-isoforms has been demonstrated in mammals [29] [30]. These GnRH-isoforms (GnRH-II, salmon GnRH and 2 forms of lamprey GnRH) most likely play a limited role in the regulation of fertility in mammals [29]. However, GnRH-II may have functions which are not related to reproduction, as suggested by the presence of high concentrations of GnRH-II in the kidneys [30], an increase in kidney weight in rats immunised against GnRH-II [29] and the widespread expression of GnRH-II receptor mRNA [29]. Immunisation

against GnRH, in order to block gonadal steroid secretion, may induce the formation of antibodies that cross-react with these GnRH-isoforms, which may result in undesired side effects due to neutralisation of these isoforms. It was shown that the cross-reactions can be circumvented by using appropriate engineered antigens [29]. Substitution of pyroglutamine at position 1 or serine at position 4 of the G6k-GnRH-tandem-dimer peptide by alanine resulted in an immunogenic antigen, which induced the formation of antibodies that neutralised only mammalian GnRH (GnRH-I) and established immunosterilisation in male piglets, without showing cross-reactivity with other GnRH-isoforms [29]. In general, isoform specific antibodies can be generated by using a peptide containing the isoform specific region, i.e. the C-terminal part of the GnRH decapepide, while the N-terminal part of the decapeptide, is altered by introducing a non-native amino acid.

GnRH immunisation in pigs

Efficacy and side effects of Specol and alternative adjuvants

The choice of the adjuvant is limited by the side effects induced. Strong adjuvants, in particular water-in-mineral oil emulsions, may cause strong and persistent inflammatory reactions. In both human and veterinary field, the risks of adjuvanted vaccines must be compensated fully by the benefits of the treatment (EMEA ´note for guidance´ CVMP/IWP/043/97). Although high immune responses were generated with the G6k-GnRH-tandem-dimer in Specol adjuvant, local reactions at the site of injection were noted. In a comparative study in young male piglets, various types of adjuvants were evaluated for efficacy and side effects (unpublished data). The following adjuvants were studied: Specol (water-in-mineral oil emulsion; ID-DLO, Lelystad, The Netherlands), Suvaxyn O/W (mineral oil-in-water emulsion; Fort Dodge Animal Health, Weesp, The Netherlands), SL-CD/Squalane, which is an aqueous formulation comprising sulfolipocyclodextrin and squalane (kindly provided by Fort Dodge Animal Health, Weesp, The Netherlands), and Carbopol 934P, comprising polyacrylic acid polymers (BFGoodrich, USA). Antigen in phosphate buffered saline (PBS) served as a negative reference. Immunisation with Specol or SL-CD/Squalane resulted in reduced testis weights and high antibody titres in all animals (Table 1). Suvaxyn was less effective, though testis growth was inhibited. The antigen administered in Carbopol and PBS, reduced testis weights in some animals.

Adjuvant	No. of responding pigs per to no.	Testis weight responding pigs, median (range)	Testis weight non-responders, median (range)	Antibody titre in GnRH ELISA[$], (mean ± SD).
Specol	10/10	25 (11-95)	-	3.0 ± 0.2
Suvaxyn	9/10	60 (22-159)	324*	1.9 ± 0.3
SL-CD/Squalane	10/10	31 (15-73)	-	3.1 ± 0.3
Carbopol	2/10	63 (30-96)	282 (248-363)	1.4 ± 0.2
PBS	1/10	67	282 (243-355)	1.3 ± 0.5

[$]Antibody titre of sera obtained 3 weeks after the booster vaccination was calculated as the log of the dilution factor which gave an optical density of 1500

* Weight of the descended testis of a cryptorchid boar.

Table 1. Efficacy of G6k-GnRH-tandem-dimer OVA conjugate in different adjuvants

Examination of the injection sites after autopsy revealed that the majority of the injections sites (17 out of 20) of Specol treated pigs showed lesions, consisting of fibrous tissue formation, granulomatous inflammation and in some cases a purulent inflammation or abscess formation. In the Suvaxyn as well as in the SL-CD/Squalane treated pigs, 60% of the injection sites could not be localised due to the absence of any tissue lesions. The lesions noted were much smaller than in the Specol treated pigs and consisted of connective tissues located intermuscularly. In the remaining groups no tissue reactions were observed. These data indicate that G6k-GnRH-tandem-dimer OVA conjugate in Specol is an effective vaccine but elicits adverse reactions. These side effects can be circumvented by using Suvaxyn or SL-CD/Squalane as adjuvant, while the desired biological effects are largely maintained. In conclusion, G6k-GnRH-tandem-dimer OVA conjugate is highly immunogenic and allows the use of a broad range of adjuvants to effectively immunosterilise male piglets.

Alternative for surgical castration

Worldwide, every year hundreds of millions male pigs are surgically castrated. Castration is performed to prevent the occurrence of boar taint, an unpleasant odour which becomes noticeable when the

meat is heated. During the last decade, politicians, veterinarians, scientist and animal welfare organisations in Europe have argued against this animal unfriendly method of surgical castration. It is painful for the animals and causes stress [29] [31]. Moreover, surgical castration affects the health of the pigs, it suppresses the immune system [29] and accounts for a doubling of the incidence of pneumonia, pleuritis and pericarditis at slaughter [15] [29]. Alternatives for surgical castration all have major disadvantages and are not likely to be implemented in the near future. Vaccination against GnRH provides an alternative, which circumvents the animal unfriendly aspects of surgical castration. In addition, it improves growth performance [5] [9] [32] making this approach even more profitable for the farmer.

GnRH immunisation improves growth performance in pigs

Several studies have demonstrated improved growth performance of pigs immunised against GnRH as compared to surgically castrated pigs [5] [9] [32]. Due to the presence of androgens during a substantial part of the fattening period, growth performance characteristics of boars vaccinated against GnRH are similar to intact boars until androgen levels become severely reduced and effective immunosterilisation apparent. It was shown that immunosterilised boars had a higher growth rate and grew more efficiently than surgically castrated pigs, while carcass quality, i.e. back fat thickness and meat percentage, was similar to intact boars. In addition, immunosterilised boars could easily be distinguished from intact boars at the time of slaughter by the size of the testes and the appearance of the scrotum. Testis size was reduced by 25% and the immunosterilised pigs exhibited a flat scrotal sac, while the scrotum of intact boars had a bulbous appearance. Moreover, testis weight of the immunosterilised boars was reduced by 75% as compared to intact boars.

In our study, feed intake was restricted and similar for all animals [29]. In case pigs have ad libitum access to the feed, effectively immunosterilised pigs show a similar eating pattern as surgical castrates [32]. Due to this high feed intake, more fat tissue is gained, resulting in lower carcass quality [32]. For this reason surgically castrated pigs are generally fed restrictedly in the last weeks of the fattening period. This feeding regimen should also be applied to boars, immunosterilised relatively early during the fattening period to prevent the development of carcasses with a high fat thickness. Feed efficiency (i.e. kg weight gain/kg feed) in boars effectively immunosterilised late in the fattening period (6 weeks before slaughter) was higher than in early immunosterilised boars (effectively immunosterilised more than 6 weeks before slaughter) and surgical castrates. Boars which received the second immunisation 2 weeks before slaughter, showed a feed efficiency similar to intact boars [5]. However, immune responses in some immunised pigs were rather low, indicating inefficient immunosterilisation. Furthermore, when the second vaccination is given within 4 weeks before slaughter, testis weight can not be used as an indication for effective immunosterilisation, as it overlaps with the testis weights in intact boars [5] [9].

Although the most important reason to ban surgical castration in piglets is the improvement of animal welfare, the better growth performance of immunosterilised boars as compared to surgical castrates could also be an important additional aspect for the implementation of GnRH immunosterilisation in pig husbandry.

Concerns about practical application of GnRH vaccination in piglets

Technically, vaccination against GnRH provides an attractive alternative for surgical castration in pigs: it effectively reduces the levels of both boar taint and it does not affect the well-being of the animal, as surgery is abolished. However, in the past decade in the Netherlands some reports/papers have been published in which the authors exhibited their concerns about the possible implementation of immunosterilisation through vaccination against GnRH in pig husbandry. The main issues of these papers are summarised below. In paper 1, vaccination against GnRH was considered as less painful for the piglet as compared to surgical castration [29]. However, no differentiation between vaccination against GnRH and surgical castration with respect to the integrity of the piglet was made; according to the author, vaccination against GnRH is in conflict with the integrity of the animal in a similar way as surgical castration, due to the fact that the animal loses its reproductive capacities.

In paper 2, it was mentioned that immunisterilisation interferes with the biological system of the pig and thus affects the ´pig-being´ (in Dutch: ´het varken-zijn´) of the pig [29]. According to the author, approval of immunosterilisation would be a step further towards the pig being ´a thing´ instead of an animal (in Dutch: ´de verdinglijking van het dier´). Furthermore, this author mentioned that immunosterilisation most likely will not be accepted by the consumer, as it will be associated with the application of hormones in pig husbandry. The Netherlands Royal Veterinary Society based their statement regarding surgical castration of pigs on the same aspects: integrity of the animal and consumer acceptance. The Society concluded that surgical castration as well as vaccination against GnRH should be rejected, while more effort should be put into alternatives [29].

Others, who considered GnRH vaccination as a suitable alternative for surgical castration without anaesthesia, argued against this attitude [29]. Moreover, the Federation of Veterinarians of Europe concluded that vaccination against GnRH could be a socially viable alternative for surgical castration, depending on the acceptance given by the consumer (FVE position paper, FVE/01/083, 2001). With respect to the ethical and social aspects of a possible implementation of vaccination against GnRH as mentioned above, the following remarks can be made: vaccination against GnRH is an animal friendly technique, in particular when compared to the current castration practice. Moreover, it causes only a temporary suppression of hormone levels of LH, FSH and gonadal steroids, while the pig maintains intact. Implementation of GnRH vaccination in pig husbandry, therefore, can be seen as a step forward, leading to a more animal friendly pig husbandry. Consumers should be informed about the current practices in pig husbandry and its consequences. Surgical castration, for instance, results in extreme high levels of LH and FSH, while immunosterilisation reduces LH and FSH levels. In this context, meat of immunosterilised pigs can be considered as 'hormone-poor' meat. Moreover, surgical castration affects the health of the pigs [15] [18]. In conclusion, objections against GnRH vaccination in pigs are not to be expected or will be at least toned down, provided that the consumer is objectively informed about this way of preventing boar taint in pigs.

Future Outlook

It is expected that the EU will approve GnRH vaccination for prevention of boar taint in the near future. It is a suitable and animal friendly alternative for surgical castration, which effectively reduces boar taint and improves growth performance.

GnRH immunisation in horses

In horses, vaccination against GnRH could be a solution for suppression of sexual behaviour in both stallions and mares. Other treatments, such as GnRH agonist or antagonist or steroid application have been shown to be inadequate, costly or prohibited at certain occasions [27], while castration carries surgical risks. However, application of GnRH vaccination in horses, is hampered by inefficiency of the vaccines [29] as well as the occurrence of side effects due to vaccination [29]. Recently, a GnRH vaccine (Equity ^{TM}, CSL Animal Health) became available in Australia to control oestrus and oestrus-related behaviour in mares. According to the medical guide, the vaccine is not effective in all mares, emphasising the difficulty to develop an effective vaccine for the use in horses.

The choice of the adjuvant is of major importance for the efficacy of the vaccine. However, it also determines the severity of the side effects. In horses, mineral oil adjuvants are effective, but these have been shown to induce severe injection site lesions [29]. It was shown that CoVaccine^{TM} HT adjuvant is well tolerated and effective in horses [29]. The vaccine reduced serum testosterone levels in all 4 treated stallions from week 6 until the end of the study (week 14). Testis function was affected as determined by reduced seminiferous tubule size, affected spermatogenesis, atrophy of the Leydig cells and a reduction in sperm quality. In a second study [29], the CoVaccine^{TM} HT vaccine reduced testosterone levels, sperm production and sperm quality for 4-5 months in 3 out of 5 treated stallions. The remaining 2 stallions responded moderate, testosterone levels in these animals were reduced for 6 weeks. The study further showed that the effects of immunisation were reversible within 8 months after the second vaccination. Despite the distinct effects on testosterone levels and testes function in young sexually mature stallions, effects may be limited in older stallions [27]. In conclusion, the use of the G6k-GnRH-tandem-dimer OVA CoVaccine^{TM} HT vaccine in sexually mature stallions is well tolerated and causes a reduction in testosterone levels, subsequently leading to affected testis function and sperm quality. However, the variation in duration of the response is a point of attention and needs to be further studied.

GnRH immunisation in humans

Chronic toxicity and efficacy study

In order to initiate a clinical trial in humans for treatment of prostate cancer, a chronic toxicity study was performed in pigs. Two candidate vaccines, containing the G6k-GnRH-tandem-dimer peptide conjugated to the carrier protein Keyhole Limpet Hemocyanin (KLH) in ISA 51 adjuvant or in CoVaccine^{TM} HT adjuvant were evaluated. Immunisation with the GnRH-CoVaccine formulations led to undetectable testosterone levels 8 weeks after the initial immunisation in all 30 animals treated. Responses in GnRH-ISA 51 treated animals were less consistent: 13 out of 30 animals showed undetectable testosterone levels at week 8 after the initial vaccination, while in 5 animals testosterone decreased at a later stage. Testosterone deficiency led to atrophy of the testes and accessory sex organs at 12, 20 and 30 weeks after the initial vaccination. After the second and third vaccination, transient local tissue reactions were seen at the site of injection in all animals treated with GnRH-

CoVaccine formulations. However, at autopsy, only minor injection site lesions were noted in some but not all animals, while lesions were moderate in GnRH-ISA 51 treated animals. Androgen depletion increased urea concentrations in plasma of surgical castrates and pigs of the GnRH-CoVaccine group, presumably due to a higher feed intake than required for maintenance and growth [29]. No effects on organ weights were observed in immunised animals and no systemic toxicological effects occurred. In conclusion, the G6k-GnRH-tandem-dimer KLH conjugate can induce a rapid reduction in serum testosterone, in particular in combination with CoVaccine adjuvant. Both vaccines were not toxic; no significant abnormalities were observed in the pigs after short-term and long-term exposure to high levels of GnRH neutralising antibodies.

Clinical trials

As mentioned, several companies have developed GnRH vaccines for treatment of gonadal steroid-dependent cancers, mainly prostate cancer, and tested these vaccines in clinical trials. However, none of the performed clinical trials have been completed successfully, despite encouraging results in animal studies. The main reason for this drawback, is a lack of full efficacy. This may be caused by the use of weak adjuvants, low antigen doses or low immunogenicity of the antigen. In addition, poor responses in men could also be caused by a compromised immunity of the patient due to age and prolonged medical treatment before GnRH vaccination was offered as a treatment [29]. We believe that the insufficient immunogenicity of the antigens described is the major problem, as suggested by the increased immunogenicity obtained with GnRH-tandem and GnRH-tandem-dimer conjugates as compared with GnRH-monomer conjugates.

Future outlook

GnRH vaccination in prostate cancer patients could be a therapy with major advantages over the currently used therapies. It does not require the use of additional medication to prevent side effects, like GnRH agonist treatment. GnRH antagonist therapy could also be a promising means to reduce androgen levels in prostate cancer patients, however high peptide doses and frequent administration are expected to be required to maintain this status. Moreover, undesired side effects may occur. Therefore, GnRH vaccination seems to be a more cost-effective and patient friendly approach for future treatment of these patients.

Overall conclusion

Vaccination against small endogenous peptides suffers from a lack of immunogenicity which can be compensated at least partially by the use of strong adjuvants. As only a limited number of adjuvants are allowed for application in practice, modification of the antigen in order to improve the immunogenicity of the vaccine is of major importance to develop an effective vaccine. We improved the immunogenicity of the small endogenous peptide GnRH by enlarging the molecule to a tandem peptide comprising twice the amino acid sequence of the native peptide. Dimerisation of this peptide and introduction of foreign amino acids further enhanced its immunogenicity. Final optimisation was established by conjugation of the optimised peptide to a efficient carrier protein. A variety of vaccine formulations, all containing the optimised GnRH-tandem-dimer, were effective and well tolerated in several mammalian species.

Perspectives

Improving immunogenicity by 'multimerisation' and amino acid substitutions has been successfully applied for GnRH and will eventually lead to a marketable vaccine. This approach can be extrapolated to many other peptides and proteins, which are of interest due to their role in tumour development and cancer. These so-called soluble mediators display a trophic effect on tumour growth by endocrine, paracrine or autocrine actions. Neutralisation of these key players by active immunisation using highly immunogenic modified antigens, may offer an appropriate and cost-effective way to block tumour growth.

References

1. Adams, T.E., Adams,B.M., 1986. Gonadotrope function in ovariectomised ewes actively immunised against gonadotropin-releasing hormone (GnRH). Biol. Reprod. 35: 360-367.

2. Awoniyi, C.A., Reece, M.S., Hurst, B.S., Faber, K.A., Chandrashekar, V., Schlaff, W.D., 1993. Maintenance of sexual function with testosterone in the gonadotropin-releasing hormone-immunised hypogonadotropic infertile male rat. Biol. Reprod. 49: 1170-1176.

3. Bonneau M., 1987. Effects of age and live weight on fat 5 alpha-androstenone levels in young boars fed two

planes of nutrition. Reprod. Nutr. Dévelop. 27: 413-422.

4. Bonneau, M., Le Denmat, M., Vaudelet, J.C., Veloso Nunes, J.R., Mortensen, A.B., Mortensen, H.P., 1992. Contributions of fat androstenone and skatole levels to boar taint: I. Sensory attributes of fat and pork meat. Livestock Production Science 32: 63-80.

5. Bonneau, M., Dufour, R., Chouvet, C., Roulet, C., Meadus, W., Squires, E.J., 1994. The effects of immunisation against luteinizing hormone-releasing hormone on performance, sexual development, and levels of boar taint-related compounds in intact male pigs. Journal of Animal Science 72: 14-2

6. Bonneau, M., Enright, W.J., 1995. Immunocastration in cattle and pigs. Livestock Production Science 42: 193-200.

7. Claus, R., Weiler, U., Herzog, A., 1994. Physiological aspects of androstenone and skatole formation in the boar. A review with experimental data. Meat Science 38: 289-305.

8. Dowsett, K.F., Knott, L.M., Tshewang, U., Jackson, A.E., Bodero, D.A.V., Trigg, T.E., 1996. Suppression of testicular function using two dose rates of a reversible water soluble gonadotropin releasing hormone (GnRH) vaccine in colts. Aust. Vet. J. 74: 228-235.

9. Dunshea, F.R., Colantoni, C., Howard, K., McCauley, I., Jackson, P., Long, K.A., Lopaticki, S., Nugent, E.A., Simons, J.A., Walker, J., Hennessy, D.P., 2001. Vaccination of boars with a GnRH vaccine (Improvac) eliminates boar taint and increases growth performance. Journal of Animal Science 79: 2524-2535.

10. Ferro, V.A., O´Grady, J.E., Notman, J., Stimson, W.H., 1995. Immunological castration using a gonadotropin-releasing hormone analogue conjugated to PPD. Food and Agricultural Immunology 7: 259-272.

11. Finstad, C.L., Wang, C.Y., Kowalski, J., Zhang, M., Li,, M.L., Li, X.M., Xia, W.G., Bosland, M.C., Murthy, K.K., Walfield, A.M., Koff, W.C., Zamb, T.J., 2004. Synthetic luteinizing hormone releasing hormone (LHRH) vaccine for effective androgen deprivation and its application to prostate cancer immunotherapy. Vaccine 22: 1300-1313.

12. Goubau, S., Silversides, D.W., Gonzalez, A., Laarveld, B., Mapletoft, R.J., Murphy, B.D., 1989. Immunisation of sheep against modified peptides of gonadotropin releasing hormone conjugated to carriers. Domest. Anim. Endocrinol. 6: 339-347.

13. Gual. C., Garza.Flores, J., Menjivar, M., Gutierrez-Najar, A., Pal, R., Talwar, G.P., 1997. Ability of an anti-luteinizing hormone-releasing hormone vaccine to inhibit gonadotropins in postmenopausal women. Fertil. Steril. 67: 404-407.

14. Hoskinson, R.M., Rigby, R.D., Mattner, P.E., Huynh, V.L., D'Occhio, M., Neish, A., Trigg, T.E., Moss, B.A., Lindsey, M.J., Coleman, G.D., Schwartzkoff, C.L., 1990. Vaxtrate®: an anti-reproductive vaccine for cattle. Aust. J. Biotechnol. 4: 166-170, 176.

15. Kruijf, J.M. de, Welling, A.A.W.M., 1988. Chronic inflammatory disease in gilts and castrated boars. (Het voorkomen van chronische ontstekingen bij gelten en borgen.) Tijdschr. Diergeneesk.. 113: 415-417.

16. Kumar, N., Savage, T., DeJesus, W., Tsong, Y.Y., Didolkar, A., Sundaram, K., 2000. Chronic toxicity and reversibility of antifertility effect of immunisation against gonadotropin-releasing hormone in male rats and rabbits. Toxicol. Sci. 53: 92-99.

17. Ladd, A., Tsong, Y.Y., Walfield, A.M., Thau, R., 1994. Development of an antifertility vaccine for pets based on active immunisation against luteinizing hormone-releasing hormone. Biol. Reprod. 51: 1076-1083.

18. Lessard, M., Taylor, A.A., Braithwaite, L., Weary, D.M., 2002. Humoral and cellular immune responses of piglets after castration at different ages. Can. J. Anim. Sci. 82: 519–526.

19. Matthews, R., Homer, D.B., Punter, P., Beague, M.P., Gispert, M., Kempster, A.J., Agerhem, H., Claudi.Magnussen, C., Fischer, K., Siret, F., Leask, H., Furnols, M.F.I., Bonneau, M., 2000. An international study on the importance of androstenone and skatole for boar taint: III. Consumer survey in seven European countries. Meat Sci. 54: 271-283.

20. McGlone, J. J., Hellman, J.M., 1988. Local and general anesthetic effects on behaviour and performance of two- and seven-week old castrated and uncastrated piglets. Journal of Animal Science 66: 3049-3058.

21. Meloen, R.H., Turkstra, J.A., Lankhof, H., Puijk, W.C., Schaaper, W.M., Dijkstra, G., Wensing, C.J., Oonk, R.B., 1994. Efficient immunocastration of male piglets by immunoneutralisation of GnRH using a new GnRH-like peptide. Vaccine 12: 741-746.

22. Millar, L.A., Johns, B.E., Killian, G.J., 2000. Immunocontraception of white-tailed deer with GnRH vaccine. Am. J. Reprod. Immunol. 44: 266-274.

23. Oonk, H.B., Turkstra, J.A., Lankhof, H., Schaaper, W.M.M., Verheijden, J.H.M., Meloen, R.H., 1995. Testis size after immunocastration as parameter for the absence of boar taint. Livest. Prod. Sci. 42: 63-71.

24. Robbins, S.C., Jelinski, M.D., Stotish, R.L., 2004. Assessment of the immunological and biological efficacy of two different doses of a recombinant GnRH vaccine in domestic male and female cats (Felis catus). J. Reprod. Immunol. 64: 107-119.

25. Simms, M.S., Scholfield, D.P., Jacobs, E., Michaeli, D., Broome, P., Humphreys, J.E., Bishop, M.C., 2000. Anti-GnRH antibodies can induce castrate levels of testosterone in patients with advanced prostate cancer. Br. J. Cancer 83: 443-446.

26. Singh, M., O'Hagan, D., 1999. Advances in vaccine adjuvants. Nat. Biotechnol. 17: 1075-1081.

27. Stout, T.A.E., Colenbrander, B., 2004. Suppresing reproductive activity in horses using GnRH vaccines, antagonists or agonists. Anim. Reprod. Sci. 82-83: 633-643.

28. Talwar, G.P., Diwan, M., Dawar, H., Frick, J., Sharma, S.K., Wadhwa, S.N., 1995. Counter GnRH vaccine. In: M. Rajalakshmi and P.D. Griffin (Editors). Proceedings of the Symposium on 'Male Contraception: present and future', New Delhi, November 1995: pp 309-318.

29. Turkstra, J.A. 2005. Active immunisation against gonadotropin-releasing hormone, an effective tool to block the fertility axis in mammals. PhD Thesis, Utrecht University, The Netherlands.

30. White, R.B., Eisen, J.A., Kasten, T.L., Fernald, R.D., 1998. Second gene for gonadotropin-releasing hormone in humans. Proc. Natl. Acad. Sci. USA 95: 305-311.

31. White, R.G., DeShazer, J.A., Tressler, C.J., Borcher, G.M., Davey, S., Waninge, A., Parkhurst, A.M., Milanuk, M.J., Clemens, E.T., 1995. Vocalisation and physiological response of pigs during castration with or without a local anesthetic. Journal of Animal Science 73: 381-386.

32. Zeng, X.Y., Turkstra, J.A., Jongbloed, A.W., van Diepen, J.Th.M., Guo, D.Z., Meloen, R.H., Oonk, H.B., van de Wiel, D.F.M., 2002c. Performance and hormone levels of immunocastrated, surgically castrated and intact male pigs fed ad libitum on a high and low energy diet. Livestock Production Science 77: 1-11.

33. Zhang, Y., Rozell, T.G., de Avila, D.M., Bertrand, K.P., Reeves, J.J., 1999. Development of recombinant ovalbumin-luteinizing hormone releasing hormone as a potential sterilisation vaccine. Vaccine 17: 2185-2191.