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OPINION OF THE SCIENTIFIC PANEL ON CONTAMINANTS IN THE FOOD CHAIN ON A REQUEST FROM THE EUROPEAN COMMISSION RELATED TO

HORMONE RESIDUES IN BOVINE MEAT AND MEAT PRODUCTS

Question N° EFSA-Q-2005-048

Adopted on 12 June 2007

SUMMARY

In line with the obligations as defined in Article 11a of Directive 96/22/EC as amended by Directive 2003/74/EC the Commission asked EFSA to examine new data on substances and products thereof with hormonal activity which may be used legally in Third Countries for growth promoting purposes in bovine meat production. The substances under consideration are the naturally occurring steroids, testosterone and progesterone, as well as the synthetic compounds trenbolone acetate, which has demonstrated affinity to androgen receptors, zeranol, which has a high affinity for oestrogen receptors, and melengestrol acetate, which resembles progestins. In accordance with the mandate, the Panel on Contaminants in the Food Chain reviewed the scientific literature that became available in the period between 2002 and the first few months of 2007, until drafting of the present Opinion. The Panel noted that the understanding of the complex mechanisms of action of steroid hormones is still a matter of scientific research and new insights into the complex genomic and non-genomic regulatory mechanisms controlling hormonal homeostasis in different phases of life are still emerging.

The Panel noted the availability of advanced methods of analysis with high sensitivity and reproducibility, allowing the measurement of residues of natural and synthetic hormones in animal tissues. However, no surveillance studies quantifying the amount and nature of residues in edible tissues of cattle treated with growth promoting hormones under practical conditions have been conducted in countries that have licensed the use of growth promoting hormones.

At present, epidemiological data provide convincing evidence for an association between the amount of red meat consumed and certain forms of hormone-dependent cancers. Whether or not hormone residues in meat contribute to this risk is currently unknown.

The CONTAM Panel concluded that the new data that are publicly available do not provide quantitative information that would be informative for risk characterisation and therefore do not call for a revision of the previous assessments of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) (EC, 1999, 2000, 2002).

KEYWORDS: growth promoting hormones, testosterone, progesterone, trenbolone acetate, zeranol, melengestrol acetate.

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LIST OF ABBREVIATIONS

ADI	Acceptable daily intake
AGD	Anogenital distance
AgNOR	Silver-stained nucleolar organizer regions
3α-DHP	3α-Dihydroprogesterone
5α-DHP	5α-Dihydroprogesterone
APPI	Atmospheric pressure photon ionization
AR	Androgen receptor
BMI	Body mass index
E2	17β-Oestradiol
EC	European Commision
EDC	Endocrine disrupting compound
EPA	US Environmental Protection Agency
ER	Oestrogen receptor
ESI	Electrospray ionization
FSDT	Fish-sexual-development test
GC	Gas chromatography
HR	High resolution
GnRH	Gonadotropin releasing hormone
GPH	Growth promoting hormones
JEFCA	Joint FAO/WHO Expert Committee on Food Additives
hCG	Human chorion gonadotropine
HP	High performance
HR	Hazard ratio
HSD	Hydroxysteroid dehydrogenase
HSO	Hydroxysteroid oxido-reductase
IC_{50}	Half maximal inhibitory concentration
IGF-1	insulin-like growth factor 1
IPCS	International Programme on Chemical Safety
i.m.	Intramuscular
IU	International unit
LC	Liquid chromatography
LH	Luteinising hormone
MGA	Melengestrol acetate
MNU	n-Methyl-n-nitrosourea
MRL	Maximum residue limit
MS	Mass spectrometry
M	Molar
OECD	Organisation for Economic Co-operation and Development
OJ	Official Journal of the European Union
P	Progesterone
PCNA	Proliferating cell nuclear antigen
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PG	Prostaglandin
PR	Progesterone receptor
SCVPH	Scientific Committee on Veterinary Measures relating to Public Health
SPE	Solid-phase extraction
T	Testosterone
TBA	Trenbolone acetate
TBO	Estra-4,9,11-triene-3,17-dione
WHO	World Health Organization
YES	Yeast estrogen screen
Z	Zeranol

BACKGROUND

Directive 96/22/EC[1] as amended by Directive 2003/74/EC[2] restricts the use of hormones in food producing animals and entirely prohibits the use of substances having a hormonal action for growth promotion. The measure is based on the Opinion of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) of 30 April 1999 (EC, 1999) and its reviews of 3 May 2000 (EC, 2000) and 10 April 2002 (EC, 2002) on the potential risks to human health from hormone residues in bovine meat and meat products.

The measure is provisional for substances listed in Annex III of the above-mentioned Directive, i.e. substances having oestrogenic (other than 17β-oestradiol and its ester-like derivatives), androgenic or gestagenic action. This is because the SCVPH concluded for the hormones testosterone, progesterone, trenbolone acetate, zeranol and melengestrol acetate that, in spite of the individual toxicological and epidemiological data available, a quantitative estimate of the risk to consumers was not possible. In consequence, Article 11a second paragraph of the above mentioned Directive requires that "*the Commission shall seek additional information, taking into account recent scientific data from all possible sources, and keep the measures applied under regular review with a view to timely presentation to the European Parliament and to the Council of any necessary proposals.*"

The Commission has thus asked EFSA to review all data (scientific publications and evaluation reports) on the hormones listed in Annex III of Directive 96/22/EC as amended by Directive 2003/74/EC that became available after the last review of the SCVPH in 2002.

TERMS OF REFERENCE

The Commission asks EFSA to examine new data on substances and products thereof with hormonal activity, which may be used legally in Third Countries for growth promoting purposes in beef cattle, e.g. testosterone, progesterone, trenbolone acetate, zeranol and melengestrol acetate, that became publicly available after the last assessment of potential risks to human health from hormone residues in bovine meat and meat products of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) in 2002 and to indicate whether such new data would call for a revision of the previous SCVPH reports (EC, 1999, 2000, 2002).

[1] OJ L 125, 23.5.1996, p. 3-9.
[2] OJ L 262, 14.10.2003, p.17-21.

ASSESSMENT

1. Interpretation of the Terms of Reference by the CONTAM Panel

The Commission asked EFSA to examine new data on substances and products thereof with hormonal activity, which may be used legally in Third Countries for growth promoting purposes in beef cattle. The mandate refers to testosterone, progesterone, trenbolone acetate, zeranol and melengestrol acetate. This implies that no further consideration of 17β-oestradiol, which is an active ingredient in 11 out of the 16 most commonly used growth promoting hormones (GPH) is requested. Hence, the effects of 17β-oestradiol were discussed in the current Opinion only where appropriate for comparative reasons. Hence, the current assessment aims to summarize and evaluate the recent literature addressing the natural hormones, testosterone and progesterone, and the synthetic compounds trenbolone, zeranol and melengestrol acetate with emphasis on new scientific data that have become available in the period between 2002 and early 2007.

2. Data collection

EFSA launched a call for scientific data related to legally used growth promoting hormones in bovines in the Official Journal of the European Union[3]. In addition, the call for data was published on the EFSA web site[4] directed towards industry, academics, consumer organisations, governmental institutions as well as EU Member States and the EU Parliament. A letter with the call for data was also sent to selected international organisations and was shared with the Advisory Forum of EFSA which has members from all EU Member States. The limited data received were assessed during the drafting of the opinion. In addition, the scientific literature that became available in the period between 2002 and early 2007 was reviewed by consulting PubMed, Agricola and ChemAbstacts databases.

3. Basic principles regarding the use of growth promoting hormones in agricultural practice in countries where these are authorised for use

The use of steroid hormones and hormone-like substances in various combinations with the aim to improve weight gain and feed efficiency in cattle and sheep is common legal practice in

[3] OJ C238, 29.0.2005, p.5
[4] http://www.efsa.europa.eu/en/science/data_collection/growth_hormones.html

various non-EU countries. Recommended application occurs in the form of small implants or devices, containing the active hormones, into the subcutaneous tissue of the ears. Both ears are completely discharged at slaughter (EC, 1999; Galbraith, 2002). Pharmaceutically, these implants represent slow-release devices, containing relatively large quantities of hormones, which are fractionally released over a period of several months. The synthetic compound melengestrol acetate (MGA) is added to the daily feed-concentrate supplies. A compilation of marketed products is given in Table 1.

Most of these commercial products contain different combinations of the natural hormones, oestradiol, testosterone and progesterone, and the three synthetic compounds, trenbolone (which predominantly binds to the androgen receptor), zeranol (which binds to oestrogen receptors), and melengestrol (which resembles natural progestins). According to the manufacturers' information, common amount of hormones in implants are: 36 – 72 mg zeranol, 200 mg testosterone, or 20 mg trenbolone acetate. Many of the products contain also 17β-oestradiol, in doses of 20 - 24 mg per implant[5]. For none of the implants is a withdrawal period required. MGA is given orally as a feed additive with a dose of 0.25 – 0.5 mg/per animal per day.

GPHs are normally used only in the growing (pre-pubertal) period in male, neutered or female animals that are intended for slaughter. Hence, potential adverse effects on the reproductive capacity of the target animals, effects on milk composition, and the excretion of administered hormones in milk need not be considered.

[5] For comparison: A mono-phasic contraceptive (which have generally the highest hormone dose) contains up to 0.05 mg oestradiol, and 0.1 - 1 mg of progestins. A menopause product (hormonal replacement therapy) may contain up to 2 - 4 mg oestradiol.

Table 1. Examples of anabolic implants, authorized for use in animals for slaughter outside the European Union

Registered trade name[#]	Active ingredient					To be used for	Pre-slaughter withdrawal period
	E2	T	P	TBA	Z		
Synovex S	+		+			Steer, bull	None
Synovex H	+	+				Heifer	None
Synovex Plus	+			+		Calf, bull, steer	None
Implix BM	+		+			Bull	None
Implix BF	+	+				Heifer	None
Implus S/ Steer-oid	+		+			Steer	None
Implus H/ Heifer-oid	+	+				Heifer	None
Compudose	+					Calf, steer	None
Revalor *	+			+		Calf, bull, steer	None
Finaplix H				+		Heifer	None
Ralgro					+	Steer, bull, calf, sheep, lamb	None
Ralgro Magnum					+	Cattle	None
Calf-oid	+		+			Calf	None
Component E-C	+		+			Bull	None
Forplix				+	+	Calf	None
Proferm					+	Cattle, sheep	None

E2: 17β-oestradiol or its benzoate; T: testosterone or its propionate; P: progesterone; TBA: Trenbolone acetate® and Z: Zeranol®.

* Different products with varying compositions, such as Revalor S, H, IS and IH, are authorized.

[#] MGA (melengestrol acetate) is not included as it is administered by oral route.

The synthetic compounds, zeranol, trenbolone and melengestrol were developed for use as growth promoting hormones in the late 1960s, and many of the toxicological data presented at that time no longer meet the requirements of modern (OECD) guidelines. Despite the call for data, no new additional information on toxicology or endocrine effects were submitted by third parties. For most of the compounds used as growth promoting hormones, Maximum Residue Limits (MRLs) have been established by national authorities and/or by the Codex Alimentarius Commission (see Table 3 in Chapter 8).

4. General considerations on hormonal action

Steroid hormones are essential for normal development and physiological function of most tissues. Their synthesis is under tight physiological regulation and subject to various feedback loops. In addition, they may circulate as pre-hormones and be converted to active paracrines or autocrines by steroid metabolising enzymes (e.g. aromatase, 5α-reductase, oxidoreductases, hydroxylases) directly in target tissues such as prostate, breast, and uterus. (for review see Bauman *et al.,* 2004). The metabolically derived compound may or may not be more active than the parent compound, and may possess different activity by interacting with one or more different receptor molecules.

The formation of hormone-receptor complexes is a mandatory first step in the translation of a steroid hormone signal. Therefore, at the cellular level, not only the hormone concentration, but also the number of receptors, determines the extent of hormone-receptor complexes formed and the level of the resultant signal. Consequently, alterations in receptor numbers may affect the level of a cellular response to a hormone. A number of factors, including natural steroid hormones and the synthetic adjuncts in growth promoting hormone implants, have been shown to exhibit regulatory roles on the expression levels of oestrogen (ER), androgen (AR) and progesterone (PR) receptors (see for example, Wilson *et al.*, 2002; Pfaffl *et al.*, 2002; Liu and Lin, 2004). In addition to hormone and receptor concentrations, binding affinity is fundamental to the selective hormone-receptor interaction, and any compound with sufficient receptor affinity may trigger the signal cascade. Moreover, higher affinity leads to saturation of receptor sites at lower concentrations of the ligand. Synthetic hormones (including those used as GPHs) have been shown to bind to steroid hormone receptors with equal or higher affinity than the most potent natural hormones (see for example Bauer *et al.,* 2000; Wilson *et al.,* 2002; Ankley *et al.*, 2003; Perry *et al.,* 2005).

Sensitivity and responsiveness to steroid hormones and hormone-like compounds varies considerably during different stages of life. Considering the effects of hormones on reproduction and development, particularly sensitive phases are:

The pre- and perinatal phase. Intra-uterine and/or lactational exposure to exogenous hormones and hormone-like substances, or non-normal concentrations of endogenous hormones have been associated with an impairment of sexual differentiation and functional impairments at later stages of life. For example, epidemiological data suggest that exposure to estrogenic and anti-androgenic compounds induce abnormalities in the reproductive tract, including hypospadia, cryptorchidism and reproductive dysfunction (Pryor *et al.,* 2000; Ikeda *et al.*, 2001; Mantovani, 2002; Obata and Kono, 2002; Rubin *et al.,* 2006).

Increasing evidence from animal models suggests that aberrant hormone exposure during gestation may result in changes in DNA methylation sites. These epigenetic changes are implicated in the transgenerational transmission of specific genetic traits as indicated by decreased spermatogenic capacity and increased incidence of male infertility in rodents (for review, see Anway *et al.,* 2005). Other persistent changes resulting from hormone exposure, such as effects on (endogenous) hormone signalling, and susceptibility to hormone-dependent and developmental diseases, have recently been reviewed by Wierman (2007).

The pre-pubertal phase. As the normal regulatory mechanisms, which control hormone exposure in adults, are not yet fully functional at this age, this period is particularly sensitive to small changes in hormone levels. Thus, inappropriate hormone exposure might impair or aberrantly accelerate normal reproductive physiology (see for example precocious puberty) (Partsch and Sippell, 2001; Teilmann *et al.,* 2002; Aksglaede *et al.,* 2006; Massart *et al.,* 2006; Muir, 2006).

The post-menopausal phase. During this stage of life, many of the physiological endocrine regulatory loops decrease in function. Consequently, abnormal hormone exposure may have profound effects, and may influence not only the reproductive, but also non-reproductive organs, including the cardiovascular system, bone tissue and the central nervous system. Similarly, tissues in which cellular replication is regulated by hormones may respond with abnormal cell proliferation, leading to hyperplasia, neoplasia and even cancer, for example, the increased incidence of endometrial and breast cancers associated with hormone replacement therapy in postmenopausal women) (for recent reviews see Honebrink, 2005; Genazzani *et al.,* 2007; and Lacey *et al.,* 2007).

In conclusion, as part of the normal homoeostasis, concentrations below which no biological effects can be determined, vary by several orders of magnitude, depending on the tissues, cells or functional markers employed in a bioassay system (Piersma *et al*., 2000). Therefore, it was not possible to assign with any certainty meaningful benchmark concentrations for hormones and growth promoting hormone adjuncts applicable to all phases of the physiological life cycle.

5. New data related to hazard characterization

5.1. Biological effects of oestrogens in pre- and postnatal development

One of the most common hormones used in pharmaceutical products, which are marketed as growth promoters in cattle, is 17β-oestradiol, also denoted E2. E2 is used in different combinations with other hormonal compounds in 11 out of the 16 products that are commercially available (see table 1). As mentioned in the introduction, a re-assessment of data on 17β-oestradiol is not included in the Terms of Reference of this Opinion and hence will not be addressed in detail. Data related to 17β-oestradiol will be used only on some occasions for comparative purposes.

5.1.1. Zeranol

Zeranol is derived from the naturally occurring mycoestrogen zearalenone, and is a potent oestrogen receptor agonist *in vivo* and *in vitro* (Leffers *et al.,* 2001; Le Guevel *et al.,* 2001; Takemura *et al.,* 2007; Yuri *et al.,* 2006). Its actions resemble those of oestradiol. (Leffers *et al.,* 2001). It is used alone or in combination with trenbolone acetate as a hormonal growth promoter in various products (see Table 1).

In vitro studies

Various *in vitro* studies suggest that zeranol may be as potent as 17β-oestradiol in terms of receptor affinity. In line with these effects, zeranol stimulates the proliferation of ER-dependent cell proliferation in MCF-7 human breast cancer cells (which are widely used in the assessment of estrogenic activity) and in transfected cells (Leffers *et al.*, 2001; Guevel and Pakdel, 2001; Liu and Ling, 2004).

Oestrogenicity and effects on expression of ER: In a study, aimed at evaluating the sensitivities of different tissues to zeranol with respect to steroid receptor (mRNA expression) regulation (Pfaffl *et al.*, 2003), increasing concentrations of zeranol resulted in significant up- or down-regulation of ERα and ERβ, depending on the tissue being investigated. These data suggest the presence of different and tissue-specific regulatory mechanisms.

Effects on cell proliferation and apoptosis: To determine whether zeranol might play a role in the neoplastic transformation of human breast tissue, MCF-10A cells, which neither express ER nor PR and are non-tumorigenic having all characteristics of 'normal' cells, were treated with different doses (0.1 - 100 nM) of zeranol or 17β-oestradiol for 20 days (Liu and Lin, 2004). The

doubling time assay, soft agar (colony formation) assay were used to study the effects on cell proliferation and tumorigenesis. ERβ expression was measured at the same time with PCR. Results showed that zeranol significantly decreased (by 30 – 40 %) the doubling time (i.e. increased proliferation rate) and, stimulated colony formation, which is indicative of neoplastic transformation. Moreover, it induced oestrogen receptor (ER-β) mRNA expression to the same extent as 17β-oestradiol. All these effects were observed at the lowest dose (0.1 nM) employed, and were not dose related. It was concluded that both zeranol and 17β-oestradiol can induce human breast epithelial cell transformation and can induce ERβ expression in human breast epithelial cells by long-term and low dose exposure, and that zeranol and oestradiol show similar potency in these assays. In earlier studies (Irshaid *et al.,* 1999; Lin *et al.*, 2000) it was shown that meat and serum from zeranol-implanted cattle possess heat-stable mitogenic activity in cultured human breast cells (MCF-10A and MCF-7) that was attributed to zeranol.

In a parallel study (Liu *et al.*, 2004), several stably transfected MCF-7 human breast cancer cell lines, expressing different levels of protein tyrosine phosphatase gamma (PTPγ), were used to compare the effect of zeranol with that of 17β-oestradiol. PTPγ is capable of inhibiting proliferation and anchorage independent growth and is implicated as a tumour suppressor gene; PTPγ mRNA expression is lower in cancerous than in normal breast tissues. Zeranol suppressed PTPγ mRNA levels and increased proliferation rate and colony formation in MCF-7 cells to about the same extent as 17β-oestradiol.

Zeranol and related derivatives had been shown previously to act as anti-apoptotic agents (Ahamed *et al.,* 2001) in MCF-7 breast cancer cells. A recent study indicates that zeranol may also suppress apoptosis in other cells in the body. The effects of zeranol on nitric oxide and endothelin-1 levels, apoptosis, and apoptotic enzymes in human umbilical vein endothelial cells were examined in the presence of the apoptosis inducer, hemocysteine (Duan *et al.,* 2006). Zeranol significantly antagonized the hemocysteine effects on nitric oxide synthase, *Bax* and *Bcl-2* expression in endothelial cells, resulting in decreased apoptosis.

In conclusion, these *in vitro* studies suggest that zeranol apparently is just as potent as the most potent oestrogens tested (17β-oestradiol and diethylstilbestrol (DES)) in terms of receptor affinity, receptor dynamics and oestrogen-dependent cell cycle regulation. No new studies on the metabolism of zeranol, and/or the genotoxicity of zeranol and its metabolites were located, although previous data suggested a genotoxic potential (Metzler and Pfeiffer, 2001).

In vivo studies

In three recent animal studies, the effects of zeranol on reproductive development have been addressed. For example, the effects of postnatal exposure to zeranol were studied in female rats following subcutaneous injections of 0, 0.1, or 10 mg/kg b.w. per day between 15 and 19 day of age (Yuri *et al.,* 2004). Zeranol did not affect body weight gain. At 28 days of age neither

morphological nor histological alterations were observed in the development of uterus, vagina, ovaries or mammary gland. However, vaginal opening (puberty onset) occurred significantly (approximately 5 days) earlier in females exposed to zeranol, compared to controls. In addition, zeranol treated females had a significantly increased cycle length due to prolonged oestrus. In the control, low zeranol dose, and high zeranol dose groups the number of animals with prolonged oestrus were 0 % (0/22), 59 % (13/22), and 78 % (18/23), respectively. When the animals were sacrificed at 37 weeks of age, body weight was comparable among all three groups, but females in the high zeranol dose group had a significantly increased relative uterine-ovarian weight. Moreover, in this study rats in both the zeranol exposed and the control group received an intraperitoneal injection of 50 mg/kg body weight N-methyl-N-nitrosourea (MNU) at 28 days of age. MNU is a carcinogen, which induces mammary tumours in female rats. No difference in the occurrence of mammary carcinoma was observed among the three groups (Yuri *et al.*, 2004). In a similar study in mice, prepubertal exposure (10 mg/kg per day s.c. injections) from 15 days of age resulted in comparable effects, including accelerated vaginal opening and prolonged oestrus, whereas the uterine, vaginal and mammary gland morphology remained normal (Nikaido *et al.*, 2005).

In a study in pigs zeranol was used in combination with hCG (human chorion gonadotropine) to evaluate its usefulness for synchronizing of oestrus and breeding in gilts (Trout *et al.*, 2007). Peripubertal gilts received a combination of 500 IU i.m. hCG and a zeranol ear implant (36 mg, Ralgro®)[6] on day 0. On day 42 the gilts received two injections of $PGF_{2\alpha}$ (prostaglandin $F_{2\alpha}$) at a dose of 10 mg given at an interval of 6 hours apart) to induce oestrus. Gilts in oestrus on day 44 - 58 were inseminated and pregnancy outcome was recorded. The use of zeranol implants did not increase the proportion of gilts in oestrus as compared to untreated gilts. However, the number of foetuses, foetal weight, foetal length, and foetal survival were all reduced in the zeranol treated group (Trout *et al.*, 2007). This study was not designed to investigate developmental effects of zeranol and involved zeranol treatment in combination with hCG and $PGF_{2\alpha}$. However, in contrast to hCG and $PGF_{2\alpha}$, zeranol was released from the implants during pregnancy and it is likely that it was the zeranol exposure that caused the deleterious effects upon the foetuses.

In conclusion, the new studies relating to effects of prepubertal zeranol exposure of females showing accelerated pubertal onset and prolonged oestrous in the cycling animals. No morphological changes of the uterus, vagina and mammary tissue were observed. The zeranol doses used in these rodent and pig studies were, however, considerably higher (10^2-10^4 fold) than the estimated concentrations which might be found as residues in tissues of zeranol-treated cattle.

In another study devoted to the effects of zeranol on prostate tissue (Gulbahar *et al.*, 2005), 24 Akkaraman lambs were implanted with increasing zeranol doses, of 12 mg (n = 8), 24 mg (n = 8) and 96 mg (n = 8), with eight lambs serving as controls. After 33 days, the prostate tissues of the

[6] Ralgro is one of the commercial products containing zeranol in a slow release formulation. Hence the actual dose (mg/kg b.w.) can not be defined.

lambs were stained using AgNOR (silver-stained nucleolar organizer regions) and PCNA (proliferating cell nuclear antigen) techniques. Both methods showed significant proliferative increases due to zeranol treatment. In addition, zeranol resulted in cystic dilatation and increased fluid in the prostate, hyperplasia and metaplasia of glandular epithelium, desquamation and focal infiltration of inflammatory cells, and hyperplasia and metaplasia of the epithelium of the collecting duct.

5.2. Biological effects of androgens in pre- and postnatal development

5.2.1. Testosterone

The role of testosterone in the development and maintenance of the reproductive system is well known and described in the 1999 SCVPH report, which also recognised the then emerging understanding of the critical role of testosterone in hormonal imprinting. From both rodent and ovine animal models it was known that exposure to excess (exogenous) testosterone at the critical developmental windows during foetal development permanently disrupts female reproductive function and leads to ovarian and endocrinological alterations resembling those of women with polycystic ovarian syndrome (PCOS). The latter include hypergonadotropism with selective increase in LH anovulatory cycles, and altered insulin sensitivity in the female offspring. Recent studies showed that in rodents, prenatal androgen receptor activation by testosterone application resulted in permanent de-feminization of the GnRH (gonadotropin releasing hormone) neurosecretory system, rendering it incapable of initiating GnRH surges, while accelerating basal GnRH pulse generator activity in adulthood. The authors suggested that these effects relate to an altered expression of progesterone receptors (Foecking *et al.*, 2005). From experiments with sheep, it was concluded that prenatal exposure to excessive amounts of testosterone decreases postnatal responsiveness to 17β-oestradiol inhibitory feedback of LH/GnRH secretion that contributes to the development of hypergonadotropism (Sarma *et al.,* 2005). Comparable results were presented by Unsworth *et al.*, (2005) and Recabarren *et al.,* (2005).

Exposure of prepubertal boys to low amounts of testosterone results in precocious puberty, as described in various case studies (Yu *et al.,* 1999; Kunz *et al.,* 2004; Brachet *et al.,* 2005). These reports demonstrate that despite the low prepubertal endogenous androgen levels, androgen dependent organs and physiological processes are already responsive to exogenous hormone exposure in the prepubertal child.

5.2.2. Trenbolone

Trenbolone acetate (TBA) is a synthetic steroid with an anabolic potency that may exceed that of testosterone. Trenbolone acetate is a prodrug that converts into its active form 17β-trenbolone,

which isomerises into 17α-trenbolone. 17β-trenbolone is the major form occurring in muscle tissue, whereas the 17α-epimer is the major metabolite occurring in liver and in the excreta including bile. 17β-trenbolone is assumed to exert its anabolic action via interaction with androgen and glucocorticoid receptors (Danhaive and Rousseau, 1986, 1988). Experiments with cattle tissues have shown that 17β-trenbolone binds to the androgen receptor with similar affinity as dihydrotestosterone. It also binds to the progesterone receptor with an affinity that exceeds that of progesterone. The other metabolites of TBA, including 17α-trenbolone (17α-hydroxy-estra-4,9,11-trien-3-one) and TBO (estra-4,9,11-triene-3,17-dione) show a significantly lower binding affinity to both types of receptors (Bauer *et al.,* 2000).

In vitro and *ex vivo* studies

Previous studies (Bauer *et al.,* 2000) had demonstrated that 17β-trenbolone, which is the major metabolite of TBA in muscle tissue, exhibited an affinity for human androgen receptor (AR) similar to that of dihydrotestosterone and a slightly higher affinity to the bovine progesterone receptor than progesterone itself. 17β-Trenbolone also was shown to have a higher affinity for the fathead minnow (*Pimephales promelas*) androgen receptor than the endogenous ligand, testosterone (Ankley *et al.,* 2003). In model experiments in rats it was demonstrated that 17β-trenbolone is a high-affinity ligand for the AR with an IC_{50} of about 4 nM in rat ventral prostate cytosol, and about 33 nM in cells transfected with the human AR. 17β-trenbolone induced AR-dependent gene expression in MDA-kb2 cells with a potency equal to or greater than dihydrotestosterone (the most potent natural androgenic steroid). Concentrations as low as 1 pM were found to significantly induce the androgen-dependent translocation of the AR into the cell nucleus (Wilson *et al.*, 2002).

In vivo studies

In experiments with rats it was shown that maternal trenbolone administration induced developmental abnormalities in the foetuses similar to the published effects of testosterone propionate. In these experiments pregnant rats were exposed to 0, 0.1, 0.5, 1, and 2 mg TBA per day by subcutaneous injections during gestational day 14 to 19. Maternal body weight gain was decreased in the exposed groups but the number of live pups was unaffected. Evaluation of the effects on the offspring was limited to a gross morphological examination of live pups at postnatal day 13. Exposed female pups had significantly increased anogenital distance (AGD) and a reduced number of nipples and areolas consistent with masculinisation. No gross malformations were noted in the male pups (Wilson *et al.,* 2002). No histological examinations were performed in this study. The manuscript states that evaluation of F1 offspring from this

study is still ongoing and will address other adverse effects associated with the administration of trenbolone during the period of sexual differentiation, but these data are not available yet.

Exposure of Japanese quail embryos to TBA on embryonic day four resulted in delayed onset of puberty in males and reduced male reproductive behaviour (Quinn *et al.*, 2007b), alterations in the copulatory behaviour of adult quail males (Quinn *et al.,* 2007a), and alterations in the immune system (Quinn *et al.,* 2007b). Many of these effects were irreversible, but the significance of these findings for human risk assessment remains questionable.

Effects in humans

Reports regarding the (mis)use of TBA as an anabolic agent in sports people describe multiple adverse effects, including liver cell injury with an increase in liver-specific enzymes in serum, cholestatic jaundice, peliosis hepatitis and various neoplastic lesions. Moreover, decreased endogenous testosterone production and spermatogenesis, oligospermia and testicular atrophy may be associated with the repeated use of TBA as anabolic (Bahrke and Yesalis, 2004; Maravelias *et al.,* 2005).

5.3. Biological effects of progestins in pre- and postnatal development

5.3.1. Progesterone

The major site of progesterone production is the corpus luteum, and, during pregnancy, the placenta. The biological effects of progesterone have been reviewed in detail by the WHO/IPCS programme (WHO-IPCS, 2000a). Progesterone and synthetic progestins are used pharmacologically in women in conjunction with ovulation stimulation drugs as well as during early pregnancy in cases of luteal phase dysfunction. Although results have been conflicting, some studies find an association between pregnancy-related intake of progestins and increased risk of hypospadias (congenital malformation of the urethral opening on the penis) in the male offspring (Carmichael *et al.,* 2005). It should however be noted that this was observed in relation to pharmacological doses of progestins, and as progesterone levels are normally high during pregnancy, minor additional exogenous progestagenic activity would presumably be without significant effects in the presence of a high endogenous activity, unless the synthetic progestins act at different sites and by different mechanisms. In contrast, serum levels of progestins in children and postmenopausal women are very low. Data on effects of progesterone in the prepubertal child are scarce and no new data have been identified. Likewise, no animal studies on the effects of progesterone during the postnatal development have been published recently.

In vitro studies

Recent mechanistic studies provided a more detailed insight into the biological potency of progesterone and its metabolites. For example, it is well established that progesterone not only serves as the precursor of all the major steroid hormones (androgens, oestrogens, corticosteroids) in the gonads and adrenals, but also is converted into one or more metabolites by most tissues in the body (Wiebe, 2006). In breast tissue and human breast cell lines, progesterone is metabolised via two major pathways: 5α-reduction (resulting in 5α-pregnanes) and 3- or 20-keto reduction (resulting in 4-pregnene derivatives (Wiebe *et al.,* 2000). 5α-Pregnane formation is catalyzed by 5α-reductase and the first (and most studied) product is 5α-dihydroprogesterone (5α-DHP). Conversion of progesterone to the 4-pregnene derivatives is catalyzed by 3α-, 3β-, and 20α-hydroxysteroid dehydrogenases (HSDs)[7] and the most studied of these is 3α-dihydroprogesterone (3α-DHP). *In vitro* studies with five human breast cell lines (MCF-7, MDA-MB-231, T47D, MCF-10A, ZR-75-1) have demonstrated that 5αP stimulates, whereas 3αHP inhibits, mitogenic properties. On the other hand, progesterone on its own (i.e. when its metabolism is blocked) had essentially no effect and therefore appears to serve only as a precursor or pro-hormone (Wiebe *et al.,* 2006). Moreover, the 5αP effects on cell proliferation and adhesion exceed the effects of 17β-oestradiol, even in cells that have oestrogen receptors (ER; e.g. MCF-7). Since the progesterone metabolites affect cell lines with various characteristics (ER/PR-positive or -negative, tumorigenic or non-tumorigenic, oestrogen-sensitive or -insensitive) it has been suggested that they may be general determinants of normalcy or cancer of the human mammary gland (Wiebe, 2006).

In summary, accumulating evidence shows that 5αP stimulates proliferation, inhibits apoptosis and decreases adhesion of cells by altering expression of molecules related to cell signaling, cell cycling, apoptosis, adhesion and the cytoskeleton via interaction with high-affinity, specific plasma membrane-associated 5αP-receptors. 3αHP exhibits the opposite effects via separate and specific plasma membrane-associated 3αHP-receptors (Weiler and Wiebe, 2000; Pawlak *et al.,* 2005). The *in vitro* effects are dose-dependent and the overall response of the cells exposed to the two metabolites depends on the relative concentrations of 5αP and 3αHP; a high 3αHP:5αP ratio promotes normalcy, whereas a high ratio of 5αP:3αHP stimulates changes from normal status to progression through increasing degrees of neoplasia. Since the level of each metabolite depends on the relative expression/activity of the specific 5α-reductase or HSDs, local concentrations can be modified by changes in expression of these enzymes (Lewis *et al.,* 2004). In addition to changes in *in situ* metabolism, local concentrations of the metabolites may also result from selective sequestering of metabolites. Thus concentrations of 5αP as high as 5×10^{-6} M have been measured in breast nipple aspirate fluid samples from tumorous breasts (Wiebe, 2006). Such high local concentrations need to be put into the context that *in vitro* 5αP can have significant effects at 10^{-8} M.

[7] HSDs are also denoted HSO (hydroxysteroid oxido-reductase) referring to the dual action as oxido-reductases

In terms of the potential effects of the GPH implants, the above studies suggest the importance of examining not only the parent compound but also the metabolites, as it is not known how much of the implanted progesterone is converted to 5αP in the treated animal. Earlier studies (Lin *et al.,* 1978) of progesterone metabolism in cattle using radiolabeled progesterone showed that 5αP is a major metabolite detected in muscle tissue. This implies that measurement of progesterone alone will not yield the relevant data. To date, differences in levels of 5αP disposition (and that of other progesterone metabolites) in muscle and liver between implanted and non-implanted animals have not been examined.

5.3.2. Melengestrol acetate

Melengestrol acetate (MGA) is given orally as a feed supplement and is widely used for growth promotion in beef cattle. The basis of melengestrol activity is its high affinity for the progesterone receptor (Le Guevel and Pakdel, 2001, Bauer *et al.*, 2000, Perry *et al.,* 2005), but its action has also been associated with increases in prolactin secretion, and activation of oestrogen receptors.

In various countries, MGA is licensed as a veterinary medicinal product intended for use as a contraceptive. Implants are used to suppress oestrus for a longer period of time.

In vitro studies

Previous reports showed that melengestrol acetate has a >5 fold higher affinity for the bovine progesterone receptor than progesterone itself, but only a weak affinity for the human recombinant androgen receptor. Following liver metabolism, the identified metabolites retain binding affinities for the receptor between 28 % and 85 % of that of progesterone (Bauer *et al.,* 2000). Experiments with the selective oestrogen receptor antagonist ICI (ICI182.789; fulvestrant) suggested that melengestrol binds also to the oestrogen receptor. This is in line with a recent study in which the weak estrogenic activity of melengestrol acetate was demonstrated by its ability to stimulate cell proliferation in the oestrogen-dependent MCF-7 cell line, whereas progesterone had no significant effect (Perry *et al.,* 2005).

In vivo studies

In a study by Pfaffl *et al.* (2002) the effect of melengestrol acetate implants on oestradiol and insulin-like growth factor 1 (IGF-I) levels were studied in heifers. In this study a biphasic effect of melengestrol treatment was observed with increased anabolism and increased oestradiol and IGF-I levels observed in the low-dose (0.5 mg per day) group compared to the controls. No anabolism, and low oestradiol and indifferent IGF-I levels were observed in the high-dose (>1.5

mg per day) group. No other new data addressing the effects of melengestrol acetate in animals or humans were found.

A detailed review of MGA containing products has been prepared by JECFA based on the original data presented for marketing authorization (WHO-IPCS, 2000b).

5.4. Epidemiology of hormone-dependent human cancers

Epidemiological study designs can be ranked according to increasing strength of evidence: ecologic (correlation) studies, cross-sectional, case-control, cohort studies, or intervention trials. Comparing the strength of evidence they provide, intervention trials are considered to provide the strongest evidence for a causal relationship on risk and have the lowest chance for potential bias to occur.

Epidemiological studies that directly address the risk of cancer in association with the use of growth promoting hormones in farm animals are lacking. Classic ecologic studies are unlikely to yield any significant information, due to the heterogeneous nature of hormonally active substances (including hormonally active natural plant constituents in food components, chemical contaminants, as well as therapeutic agents) that can occur in beef meats. A prospective randomized trial with many thousands of subjects, allocated to two groups, one having access only to meat from untreated animals, and the other consuming only meat from animals treated with GPHs, and with all other confounding factors (age, sex, socio-economic status, life-style variables including diet) equally distributed between the two groups, can not be achieved. A brief overview of actual data (from 2002 onwards) on hormone-dependent cancers of the breast, endometrium and ovaries in women, as well as cancers of the testis and prostate in men is presented in Annex 1. These studies include intervention trials with hormone receptor antagonists as well as inhibitors of enzymes involved in hormone synthesis, which are used at therapeutic dosages for humans and which differ in many cases from the compounds used as GPH. None of these studies includes beef consumption as a variable, and hence although these studies provide valuable mechanistic insight they are of limited significance for the evaluation of the effects of hormone residues in beef meats.

6. Exposure assessment: new data related to occurrence of residues of hormones in edible tissues

6.1. Recent developments in the analysis of residues of natural and synthetic steroids

Until the end of the 1990s, attempts to analyse low levels of steroid residues in cattle were restricted to the use of low resolution GC-MS with quadrupole mass analyser. GC-MS^n with ion trap mass filter was also used, but with some commonly reported difficulties in terms of identification and quantification performances (matrix effects and poor repeatability in terms of ion ratio in complex biological matrices). Improvement of single quadrupole (ion transmission, mass scanning) and ion-trap detectors (external ion source) resulted in the development of more sensitive analytical methods (Le Bizec *et al.*, 2004) and Hartmann and Steinhart (1997) reached detection limits of 20 to 100 ng/kg (determined on spiked samples) using GC-MS on a single quadrupole. With efficient sample purification (based on liquid-liquid extraction or solid phase extraction, semi-preparative HPLC), oestrogens, androgens and progestagens can be detected at the low level of 10 ng/kg incurred residues in muscle and kidney samples ("ISOSTER", European project[8]).

When very low limits of detection are required, GC-HRMS (double focusing sector instrument, R >10,000) remains the ultimate analytical tool. Efficient monitoring of steroid residues (from screening to confirmation) is possible in almost all matrices and especially in meat products. Meunier-Solère *et al.* (2004) developed a method that combined a selective purification procedure with high resolution mass spectrometry. The limits of quantification achieved with this technique were as low as 1 ng/kg in liver, muscle and kidney.

However GC-MS requires derivatisation of the steroids by means of silylation, acylation or oxime/silylation reaction, depending on the properties of the individual steroids. The lack of appropriate derivatisation agents, the failure of such reaction for some steroids, e.g. trenbolone, to give a single reaction product, and problems with chemical rearrangement of others, strongly stimulated the development of LC-MS based methods (Stolker *et al.*, 2005). LC-MS/MS has become widely used as a complementary technique to GC-MS in residue analysis because of its applicability to the determination of polar and/or non-volatile compounds without derivatisation (Balizs and Hewitt, 2003). Measurement, and particularly identification, of phase II metabolites (glucurono-, sulfo- and fatty acid esters) can only be performed using LC-MS. Electrospray ionization (ESI) is probably most widely used for direct measurement of phase II metabolites,

[8] EC Framework 5 project 473. Determination of the origin of hormones in cattle (ISOSTER) http://cordis.europa.eu/data/PROJ_FP5/ACTIONeqDndSESSIONeq112242005919ndDOCeq473ndTBLeqEN_PROJ.htm

whereas the more recently introduced atmospheric pressure photon ionization (APPI) is preferred for free non-polar steroids (Le Bizec *et al.*, 2004).

The analysis of the ratio of free and conjugated forms (including lipoidal esters) of natural hormones provides also the means to discriminate between residual amounts of endogenous hormones in muscle tissues and body fat, and residues resulting from the application of hormone implants used for growth promotion (Maume *et al.*, 2001). In addition, techniques based on combined GC with stable isotope MS allow the discrimination between endogenous and administered hormones.

A detailed description of these recent advances in analytical methods is given in Annex II.

6.2. New data on the occurrence of residues in edible tissue

A recent report (Paris *et al.,* 2006), in which residue levels of experimentally implanted animals were analysed with the above mentioned advanced methods, indicates significant differences between treated and non-treated animals of the same age group, for example for oestradiol residues in the liver, kidneys, muscle- and adipose tissue (Table 2). For example in the liver treated animals had oestradiol levels of 22.5 $\pm$ 6.6 versus 5.5 $\pm$ 2.4 ng/kg in the control animals. In the muscle tissue treated animals had a level of 41.3 $\pm$ 19.2 ng/kg, whereas control levels were below the limit of detection (for details see Maume *et al.,* 2001; Paris *et al.,* 2006). Hence these findings suggest that human exposure to natural hormones such as oestrogens could increase if GPH implants are used on a large scale in commercial beef production.

The levels measured for testosterone in implanted animals were also on average higher than those found in control animals, but remained in the same range or were lower than those found in intact bulls. The tissue levels of progesterone in implanted animals were in the same range of those of control animals (only a slight increase in some residue levels in the kidneys were observed), but remained below the tissue levels that occur during pregnancy, when physiological progesterone levels are very high. In the interpretation of these results in terms of exposure assessment for beef consumers it needs to be emphasized that the vast majority of slaughter animals are heifers and young steers kept for fattening (the target animals for GPHs), and not adult animals or even pregnant cows.

Residues of zeranol and trenbolone do not occur in animal tissues under normal conditions, but can be measured following the use of GPHs containing these compounds. Itshould be noted that the mycotoxin zearalenone that is present in various feed materials can be converted into zeranol. This fact might complicate the monitoring of undesirable residues in animal tissues.

Taken together, these results confirm earlier reviews of tissue levels of oestrogens, testosterone and progesterone. The new data provide additional evidence for the expected higher exposure of consumers if GPHs are used on a large scale, and without appropriate withdrawal times. The fact that commercial GPH products are distributed over-the-counter in countries where they are authorized for use in beef cattle, increases the likelihood of improper use (application of more than one implant at a given time, applications at other injection sites) which would result in higher residue levels, as already mentioned (EC, 2002).

Table 2. Concentrations of residues of hormones present in edible tissues from non- treated and treated animals (compilation of data reported by various authors; taken from Paris *et al.,* 2006 with permission).

Mole-cules	**Physiological phase**	**Main Residues**	**Tissue residues** [1]			
			Liver	**Kidneys**	**Muscle**	**Adipose tissue**
Oestra-diol	Control veal calves, heifers or steers[2]	Oestradiol 17α-oestradiol Oestradiol-esters Estrone	11 (5-53)[3] 0-5 15 (11-198)	7 (2-70) 5-10 (23-166)	5 (3-35) ND ND 6	(5-50) ND ND 23
	Implanted veal calves, heifers or steers[4]	Oestradiol 17α-oestradiol Oestradiol-esters Estrone	50 (5-1650)[3] 220 (20-4158) (20-25) (57-284)	47 (6.4-589) 133 (2-5) (34-144)	25 (11-280) ND ND (3-72)	40 (9.3-358) (1-3) 35 (29-149)
	Cows: - follicular phase - luteal phase	 Oestradiol Estrone Oestradiol Estrone	 23 14.3	 13.5 15.1	 30.8 18.9	 11 28.4 8 25.9
	Pregnant cows: - 120 days - 240 days	 Oestradiol Estrone Oestradiol Estrone	 13.3 18.2 32.7 145	 82.5 85.3 1027 142	 118 156 274 523	 48.1 1283 67.5 2786
Zeranol	Implanted veal calves, cows or steers	Zeranol	0.35-1.21	0.08-0.22	0.01-0.73	0.06-0.22
Testo-sterone	Control veal calves or heifers	Testosterone	0.021-0.126	0.043-0.356	0.006-0.029	0.021-0.296
	Implanted veal calves or heifers		0.016-0.196	0.228-0.588	0.031-0.360	0.032-1.258
	Bull		0.75	2.78	0.54	10.95
	Pregnant cows: - 120 days - 240 days		 0.05 0.27	 1.51 4.01	 0.27 0.42	 0.59 0.69
Tren-bolone	Implanted steers	Trenbolone Trenbolone-17α	0.19-0.76 0.80-4.02	0.13-0.39 0.12-0.18	0.18-0.28 0.01-0.02	0.26-0.85 0.03-0.06
Progest-erone	Control veal calves or heifers	Progesterone	0.16-0.75	0.03-4.07	0.0-0.9	0.87-1.60
	Implanted veal calves or steers		0.16-0.92	0.11-2.80	0.23-0.77	3.20-8.66
	Pregnant cows		3.4	6.2	10.1	239.0

[1] Residues (mean values) in tissues are expressed in ng/kg (ppt) for oestrogens and in μg/kg (ppb) for the other compounds found in different independent studies.

[2] Sum of free and conjugated oestrogens

[3] In brackets, minimal and maximal values

[4] Cattle can be implanted with products containing an androgen-like substance (trenbolone acetate or testosterone) and an oestrogen-like substance (oestradiol or zeranol) at different physiological stages, either as milk-fed veal calf or later as steer, heifer or even as the adult cow. In all these cases, the hormone dose in the implant differs for calves, steers or heifers.

7. Environmental impact of large-scale use of growth promoting hormones in beef production

A large group of chemically diverse compounds has been identified with the potential to alter the normal functioning of the endocrine system in wildlife and experimental animals (Vos *et al.,* 2000; Daston *et al.,* 2003). These compounds are denoted as Endocrine Disrupting Compounds (EDC). According to their mechanism of action, zeranol, trenbolone and melengestrol acetate have been included as EDCs in the WHO-IPCS assessment (WHO-IPCS, 2002 and related documents cited in this review).

Quantitative data on the overall use of GPHs are not available, and many reports focus on regions with a dense cattle population. For example, Soto *et al.* (2004), describe significant estrogenic and androgenic activity released into the water by beef feedlot operations in Nebraska (USA). Using cell-based reporter assays, such as the E-screen and A-screen method (the E-screen detects estrogenic activity, the A-screen androgenic activity), sufficient levels of hormonally active compounds to produce adverse effects on aquatic ecosystems were found. These findings were supported by Orlando *et al.* (2004), reporting male fish (fathead minnow) de-masculinisation (lower testicular testosterone synthesis, altered head morphometrics, and smaller testis size) and female de-feminisation (decreased oestrogen:androgen ratio) in river water that contained effluents from cattle feedlots[9]. The androgenic activity of 17β-trenbolone in fathead minnow, lowering fish fecundity, reducing plasma steroid (testosterone and 17β-oestradiol) and vitellogenin concentrations had been described before (Orlando *et al.*, 2004; Ankley *et al.,* 2003; Wilson *et al.*, 2002) and has also been reported in mosquito fish (Sone *et al.*, 2005; Jensen *et al.,* 2006). In a recently developed fish-sexual-development test (FSDT), significant effects of trenbolone acetate at concentrations exceeding 193 ng/L were observed, shifting a zebra fish population towards a dominance of males and decreasing vitellogenin levels significantly (Holbech *et al.*, 2006).

The findings by Orlando *et al.* (2004) have been corroborated by Durhan *et al.* (2006), who identified metabolites of trenbolone acetate (17α- and 17β-trenbolone) in a study assessing water samples from a beef feedlot in Ohio known to use trenbolone acetate implants (Durham *et al.*, 2006). The samples for this study were collected in 2002 and 2003 and were subjected (in addition to the chemical analyses) to a bioassay using CV-1 cell line (transiently co-transfected with human androgen receptor vector), which indicated an elevated level of androgenic activity.

Comprehensive reviews on the environmental effects of androgens found in effluents from animal feedlots, specifically mentioning the Nebraska site and the Ohio site have been published by Gray *et al.* (2006), and Mill and Chichester (2005). Khanal *et al.* (2006) estimated that in

[9] Experimental data had already indicated that synthetic androgens like trenbolone and its metabolites are stable in cattle manure over a period of more than 4.5 months (Meyer, 2001).

various regions about 90 % of the oestrogen load of surface waters results from animal manure of concentrated feeding operations. These authors also showed that significant differences exist in the degradation of 17β-oestradiol (rapidly degraded) and estrone (poorly degraded), indicating the necessity to test the environmental fate of individual compounds. It is noteworthy that in consideration of these findings, the US EPA[10] prescribed managerial measures to protect the country's water quality.

In the UK (in which the use of GPHs is prohibited), the contamination of freshwaters from effluents of livestock farms has recently been assessed (Matthiessen *et al.,* 2006). Total estrogenic activity was determined using the Yeast Estrogen Screen (YES), and in parallel, estrone, 17β-oestradiol and 17α-ethinyloestradiol were analysed by LC-MS/MS. The same group of authors assessed the potential contribution of farm animal operations to the total environmental oestrogen burden. They concluded that in the UK, farm animals are responsible for 15 % of all the oestrogens in surface water at or downstream of livestock premises. However, given the rapid biodegradation and high sorption rates, less than 0.001 % of oestrogens would reach the field drains (Johnson *et al.,* 2005).

8. New data related to risk characterization

JECFA has proposed ADI values and maximum residue limits for a number of the compounds under consideration (see Table 3). In the absence of residue data from countries in which the use of growth promoting hormones is authorized, these values cannot be linked to the actual exposure of consumers.

[10]In February 2003, the US Environmental Protection Agency (EPA) issued new legislation, National Pollutant Discharge Elimination, System Permit Regulation and Effluent Limitation Guidelines and Standards for Concentrated Animal Feeding Operations (CAFOs), requiring that CAFOs take appropriate actions to manage manure effectively in order to protect the country's water quality. This new legislation revised and set requirements for CAFOs under the Clean Water Act.

Table 3. ADIs and MRLs proposed by international organisations or by countries using growth promoting hormones.

Substance	ADI (μg/kg b.w.)	MRL in μg/kg	Proposed or established by
Testosterone	0-2	No MRLs necessary	JECFA/Codex
		Only natural levels permitted: In uncooked edible tissues of heifers: 0.64 in muscle, 2.6 in fat, 1.9 in kidney, 1.3 in liver.	USA
		No limits in animal products.	Australia
Progesterone	0-30	No MRLs necessary	JECFA/Codex
		Only natural levels permitted: In uncooked edible tissues of steers and calves: 3 in muscle, 12 in fat, 9 in kidney, 6 in liver. In uncooked edible tissues of lambs: 3 for muscle, 15 for fat, kidney, and liver.	USA
Trenbolone acetate	0-0.02	Muscle: 2 (as β-trenbolone); liver: 10 (as α-trenbolone)	JECFA/Codex
	0.4	No tolerance needed	USA
	0.02	Administrative MRLs: Cattle: muscle 2. liver 10	Canada
		Cattle: muscle: 2, liver: 10	Japan
Zeranol	0–0.5	Bovine: 2 muscle, 10 liver	JECFA/Codex
	1.25	Cattle: no tolerance needed, sheep: 20 in edible tissue	USA
		Administrative MRLs: Cattle: muscle 2. liver 10	Canada
		Cattle: muscle 2. liver 10	Japan
		Cattle: meat 5, edible offal 20	Australia
Melengestrol acetate	0–0.03	Suggested by JECFA but not adopted by Codex. Cattle: liver 2, fat 5	JECFA
		A tolerance of 25 for residues of melengestrol acetate in fat of cattle	USA
		Administrative MRLs. Cattle: liver 6, fat 14	Canada

Sources:

JECFA: Summary of Evaluations Performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA 1956-2005) (First through Sixty-fifth Meetings). Website consulted April 2007. http://jecfa.ilsi.org/search.cfm

Codex: Codex Alimentarius commission. Veterinary Drug Residues in Food. Maximum Residue Limits. Website consulted April 2007. http://www.codexalimentarius.net/mrls/vetdrugs/jsp/vetd_q-e.jsp

US: Code of Federal Regulations (CFR). Main Page. Webpage consulted April 2007. http://www.gpoaccess.gov/cfr/index.html

Canada: Health Canada. Administrative Maximum Residue Limits (AMRLS) and Maximum Residue Limits (MRLS) set by Canada. Website consulted April 2007. http://www.hc-sc.gc.ca/dhp-mps/vet/mrl-lmr/mrl-lmr_versus_new-nouveau_e.html

Australia: Food Standards. Australia New Zealand Food Standards Code. Website consulted April 2007. http://www.foodstandards.gov.au/thecode/foodstandardscode.cfm

Japan: The Japan Food Chemical Research Foundation. Maximum Residue Limits (MRLs) List of Agricultural Chemicals in Foods. Webpage consulted April 2007.

http://www.m5.ws001.squarestart.ne.jp/foundation/search.html

Epidemiological studies that would have the potential to indicate an association between the consumption of meat from GPH treated animals and health risks are lacking, and will most likely not become available due to methodological obstacles (see 5.4.) In contrast, several hundred papers have been published in the period between 2002 and 2006 (the period under consideration) on the topic of meat consumption and cancer, confirming earlier studies with stronger data and incriminating more cancer sites than previously. For example, the influence of red meat intake on the occurrence of pre-menopausal breast cancer was evaluated in the Nurses' Health Study II. A higher red meat intake was strongly related to breast cancer risk for oestrogen and progesterone receptor positive tumours, with a dose response relationship going up to HR (hazard ratio) values of 1.97 (1.35 - 2.88) for more than 1.5 servings per day, compared to 3 or less per week (Cho *et al.,* 2006). In the discussion of these findings, the authors presented a number of possible factors that may account for this increased incidence of cancer, which include, among others, the consumption of meat derived from cattle treated with hormones. Other risk factors are the formation of carcinogens such as heterocyclic amines, *N*-nitroso-compounds and polycyclic aromatic hydrocarbons during cooking and processing, enhancement of oestrogen-induced tumour formation by heme iron present in meats, and the overall fat intake.

The most recently published study on meat consumption and breast cancer has been conducted in the United Kingdom (where growth promoters have been banned since 1988), in the UK Women's Cohort Study (Taylor *et al.*, 2007). The risk of breast cancer was evaluated with an initial collection of data on diet and prospective follow-up. Data were corrected for common confounding factors such as high alcohol consumption and/or smoking. Statitical analyses revealed that both, pre- and post-menopausal breast cancer, were associated with meat intake with dose-response trends in all groups. Red meat and processed meat showed the most pronounced effects, with HRs of up to 1.7 (1.2 - 2.4) for high consumption of processed meat compared to none. In general highest *versus* lowest tertile of intake for various meats were associated with HR of the order of 1.3.

Whilst previous studies focussed on a relation between meat consumption and cancer, a very recent study in the USA, comparing mothers' recollections of their meat consumption during pregnancy with the sperm count of their adult sons, suggested that meat consumption might affect human fertility (Swan *et al.,* 2007). Additional investigations in other countries with different consumption habits are necessary to establish whether this association is causal.

It has been suggested that GPHs may have played a role in some of the above observations, but currently there is no scientific evidence to link these effects with residual amounts of GPHs that might be present in meat.

CONCLUSIONS

- New data published since 2002 confirm and extend the current understanding of the effects of steroid hormones and hormone-like substances used as growth promoting hormones (GPH), which are mediated not only via interactions with their specific receptors.

- In *in vitro* systems, the potencies of zeranol, trenbolone and melengestrol acetate in terms of oestrogen, androgen and progesterone receptor affinities and modulation of gene expression, as well as cell proliferation and apoptosis, may be equal to, or exceed, those of the most active natural hormones. There is a lack of information with respect to the *in vivo* significance of these effects at exposure levels associated with residues in meat.

- Sensitive analytical methods have become available permitting the identification and quantification of the growth promoting hormones (all five compounds under consideration) and their currently known major metabolites. Regarding the natural hormones, testosterone and progesterone, these methods also allow discrimination between endogenous and exogenous hormone residues. These advanced methods have as yet, only been used in a very limited number of experimental studies, and await to be applied on a broader scale.

- In the absence of data from surveillance studies, the exposure to residues of the hormones used as growth promoting agents cannot be quantified. In particular, the available data on the metabolism of trenbolone, zeranol, or melengestrol acetate in cattle, and the amount and nature of residues in animal tissues following routine use of these compounds in beef cattle operations, are too incomplete to be assessable.

- An increasing number of publications presenting epidemiological data indicate a correlation between red meat consumption and hormone-dependent cancers of the breast and prostate. Due to the high number of confounding factors, the contribution of residues of hormones in meat cannot be quantified in these studies.

- Large-scale cattle production and the use of growth promoting hormones in cattle operations in Third Countries has been associated with undesirable effects in sentinel aquatic species in contact with cattle farm effluents.

The CONTAM Panel concluded that the new data that are publicly available do not provide quantitative information that would be informative for risk characterisation, and therefore do not call for a revision of the previous assessments of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH).

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ANNEX I. HORMONE-DEPENDENT CANCERS

Among the hormone-dependent cancers, breast cancer, endometrial and to a lesser extent ovarian cancer, demonstrate the highest age-standardized incidence rates in North America, Western Europe and Australia-New Zealand. In particular exposure to hormonally active compounds during childhood and puberty seem to predispose for hormone-dependent cancers (Sasco *et al.*, 2003), but neither a qualitative (enumeration of estrogenic compounds in the total diet) nor a quantitative exposure assessment can be presented, due to the large differences in dietary habits in these geographic regions. According to the IARC Globocan data, the most recent of which refer to 2002 (Parkin *et al.,* 2005), the global pattern of cancer incidence remains unchanged.

I-1. Hormone-dependent cancers in females

I-1.1. Breast cancer

Breast cancer and oestrogens

Several thousand references are available on the topic of hormones and breast cancer. The majority refer to oestrogens (oestradiol, estrone, estriol and their metabolites). Women with the highest endogenous levels have the highest breast cancer risk and the use of oral contraceptives as well as hormone replacement therapy for menopause are associated with an increased risk of breast cancer (IARC, 1999). In addition, a number of studies have addressed the issue of mechanism of action of oestrogens, demonstrating that in addition to hormonal effects, oestradiol through its metabolites may exert genotoxic effects. For example, it could be shown that one of the metabolites (oestradiol-3,4-quinone) induces specific (A-T to G-C) mutations in the DNA of the rat mammary gland. These findings reinforced previous findings on the specific metabolic pathways of oestrogens, and suggesting a genotoxic action of oestrogens, independent of and complementary to the hormonal action (Mailander *et al.,* 2006).

Breast cancer and testosterone

Numerous studies have evaluated the role of endogenous levels of androgens, mostly testosterone and androstenedione in breast cancer risk, and only the most recent ones are mentioned here. Almost all available studies are prospective cohort studies where the hormonal determinations have usually been done years in advance of the diagnosis. A higher risk of breast cancer has been found for high testosterone levels, regardless of predicted risk or family history of breast cancer in a prospective nested case-control study within the Nurses' Health Study (Eliassen *et al.,*

2006a). Detailed results on hormonal levels in premenopausal women showed that testosterone and androstenedione were associated with only a modest increase in risk of breast cancer, becoming statistically significant for invasive and ER^+/PR^+ cancers (Eliassen *et al.,* 2006b). Among post-menopausal women of the same cohort, risk was evaluated for postmenopausal hormone users and a modest association was found in relation only to free oestradiol and free testosterone (Tworoger *et al.*, 2005). This is slightly different from results previously reported by the same team (Hankinson *et al.*, 1998). Similar results were found in a Hungarian case-control study, with an increased risk of post-menopausal breast cancer for the highest quartile of testosterone particularly marked for ER^+/PR^+ tumours (Kahan *et al.,* 2006). A prior cohort study in the USA had found that high testosterone levels predicted ER^+ breast cancer (Cummings *et al.,* 2005). This influence of free testosterone levels on breast cancer risk was also described before in a prospective case-cohort study in the USA (Cauley *et al.,* 1999). In contrast, in a case-control study of post-menopausal breast cancer in Michigan, USA, testosterone and other androgen levels were not associated with risk (Adly *et al.,* 2006). Similarly, an ancillary study within the NSABP Breast Cancer Prevention Trial (P-1) found no support for the use of endogenous sex hormone levels to identify women who are at particularly high risk of breast cancer (Beattie *et al.*, 2006). In a case-control study nested within the European EPIC cohort study and conducted among post-menopausal women, elevated androgen levels were associated with breast cancer risk with values of the odds ratio (OR) as estimates of the relative risk (RR) of 1.85 for testosterone and 2.50 for free testosterone at the highest quintile (Kaaks *et al.,* 2005a). Similar results were found for pre-menopausal women (Kaaks *et al.,* 2005b). The EPIC study also evaluated hormonal levels in healthy women, looking among others at the correlation between IGF-1 and increased androgen levels (Bezemer *et al.,* 2005), the correlation between C-peptide and free testosterone (Verheus *et al*., 2006), high SHBG level and mammographic density (Warren *et al*., 2006), higher alcohol intake and higher testosterone level (Rinaldi *et al.,* 2006b), and adiposity (Rinaldi *et al.,* 2006a). Other researchers also considered racial/ethnic differences (Setiawan *et al*., 2006), body mass index (BMI) (Lukanova *et al*., 2004, Lamar *et al*., 2003), BMI and physical activity (McTiernan *et al*., 2006) or oral contraceptive use (Hietala *et al*., 2007). In a study limited to breast carcinoma in situ and conducted as a case-control study nested within a prospective cohort of the New York University Women's Health Study, no association was found for increasing levels of any hormone (Zeleniuch-Jacquotte *et al*., 2005). This result is in contrast to the majority of previous studies which describes consistently an increased risk of breast cancer for increasing endogenous levels of androgens and in particular free testosterone, in Sweden (Manjer *et al*., 2003), the Netherlands (Onland-Moret *et al*., 2003), China (Yu *et al*., 2003) and Egypt (Abu-Bedair *et al*., 2000). A study conducted in the USA found a more than doubled risk for each on the following hormones: oestradiol, testosterone, androstenedione, and dehydroepiandrosterone sulfate, with associations being stronger for *in situ* disease (Missmer *et al.,* 2004). Yet, a study of breast dysplasia did not reveal an association with androgens, but a strong and significant correlation with oestrogens (Schairer *et al.,* 2005). An increased risk associated with increased levels of all sex hormones was also found when nine prospective studies among postmenopausal women were re-analysed (Key *et al*., 2002).

The influence of testosterone levels, either by themselves (Berrino *et al*., 2005) or as part of the metabolic syndrome (Pasanisi *et al*., 2006) has been evaluated and found to increase the risk of recurrence of breast cancer.

Few studies are available on the influence of exogenous testosterone use in women. The prospective Nurses' Health Study assessed the risk of breast cancer according to the types of postmenopausal hormone formulation. Among women with natural menopause, the risk of breast cancer was 2.48 times greater among current users of oestrogen plus testosterone compared to never users. Among menopausal hormone users the risk is significantly greater for the combination of oestrogen and testosterone as compared to oestrogen alone, and marginally greater than for the combination of oestrogen and progesterone (Tamimi *et al*., 2006). This is in contrast to a previous Australian study that concluded that the addition of testosterone to hormonal therapy for menopause did not increase the risk of breast cancer (Dimitrakakis *et al*., 2004).

Breast cancer and progesterone

The number of studies addressing the potential influence of endogenous progesterone levels on breast cancer risk is much lower than the number of studies devoted to oestrogens or even androgens. This may be explained by the fact that progesterone was for a long time considered a benign molecule with positive impact, and without many major side effects.

This assumption was to a certain extent confirmed by a case-control study nested within the EPIC cohort where elevated serum progesterone concentrations were associated with a statistically significant reduction in breast cancer risk with an OR at 0.61 for the highest quartile (Kaaks *et al.*, 2005b). A similar result had already been published based on an Italian cohort (Micheli *et al*., 2004).

The interest in progesterone, and more generally in the progestins, increased when they were used in postmenopausal hormonal replacement therapy regimes. Based on many large and convincing epidemiological studies, the International Agency for Research on Cancer (IARC) upgraded the classification of carcinogenicity from 2B (possible carcinogen) to 1 (recognized carcinogen) for both oestrogen-progestagen post menopausal therapy and for oestrogen-progestagen oral contraceptives (IARC, in press). Examples for studies that were considered in this evaluation are the Women's Health Initiative, which attracted broad attention. This study was a randomised controlled trial of oestrogen and medroxyprogesterone acetate combinations compared with placebo treatments in healthy post-menopausal women. The trial was interrupted in 2002, because of the high breast cancer incidence, with a statistically significant increased hazard ratio of 1.26 (Roussow *et al*., 2002). The results published a year later suggest that oestrogen-progestin combinations stimulate breast cancer growth and hinder breast cancer diagnosis (Chlebowski *et al.*, 2003). Similar results were obtained in Denmark (Stahlberg *et al.*,

2003). The risk was found to be greater for oestrogen-progestin combinations than for oestrogen alone in several observational studies, including the Women's Health Study in the USA (Porch *et al*., 2002), and other studies in the USA (Li *et al*., 2003), Sweden (Jernstrom *et al*., 2003), Norway (Bakken *et al*., 2004), France (Fournier *et al*., 2005), UK (Beral *et al*., 2003). A few studies did not find a difference due to type of treatment (Ewertz *et al*., 2005) and it was hypothesised that androgenic progestins rather than progesterone itself account for the increased risk (Campagnoli *et al*., 2005; McCarty, 2001). Progesterone alone is capable of inhibiting human breast cancer cell growth *in vitro* (Gizard *et al*., 2005).

I-1.2. Endometrial cancer

Endometrial cancer and oestrogens

Endometrial cancer is the cancer most closely linked to oestrogens. Initially, progestins were added to the treatment regime of oestrogen-replacement therapy for menopausal females to avoid an increase in the risk of endometrial cancer (Persson, 2000; Emons *et al*., 2004). Most risk factors for endometrial cancer can be explained within the so called unopposed oestrogen hypothesis, which proposes that exposure to oestrogens unopposed by progesterone or synthetic progestins leads to increased mitotic activity of the endometrial cells, higher number of DNA replication errors, and somatic mutations resulting in malignant phenotype (Akhmedkhanov *et al*., 2001).

Endometrial cancer and testosterone

Few studies are available on the correlation between endogenous hormone levels and endometrial cancer. Elevated plasma levels of androstenedione and testosterone are associated with increased endometrial cancer risk, both among pre- and post-menopausal women. Increased levels of estrone and oestradiol are also risk factors among postmenopausal women. It has been hypothesized that after the menopause, when progesterone synthesis has ceased, excess weight and obesity may result in increasing oestrogen levels through the aromatisation of androgens and androgen precursors in the adipose tissue and hence in an increased risk (Kaaks *et al.,* 2002; Lukanova *et al*., 2003). A recent case-control study nested within three cohorts in New York, Umea and Milan, found an OR at 1.74 for the highest versus the lowest quartile of serum testosterone level in postmenopausal women (Lukanova *et al*., 2004).

Multiple studies are available on mechanistic aspects of steroid hormone induced carcinogenesis. Data show that

- activation of the AKT cascade (Rice *et al*., 2006),

- CYP17 genetic polymorphism (which may for the A1/A1 CYP17 variant be associated with non steroidal pathways (Bernstein *et al*., 2002)),
- expression of androgen receptors and 5α reductase (converting testosterone into the more active 5α-dihydrotestosterone in the endometrium) (Ito *et al*., 2002),
- activity of the tumour-suppressing pro-apoptotic regulator PAR4 (Garcia-Cao *et al.*, 2005),
- activity of 17β-hydroxysteroid dehydrogenase (regulating the in situ oestrogen-production in endometrial carcinomas (Ito *et al*., 2006), as well as
- activity of CYP450 aromatase (site specific conversion of androgens into mitogenic oestrogens (Jongen *et al*., 2005),

may influence the risk. The role of CYP19 haplotypes has been studied in a nested case-control study within the Nurses' Health study and a high frequency haplotype was found to be associated with higher oestrogen:androgen ratios and increased risk of endometrial cancer, in particular among postmenopausal women (Paynter *et al*., 2005). In contrast, an earlier study had found no evidence for a role for PPARγ or Pro12Ala polymorphisms in endometrial cancer susceptibility (Paynter *et al*., 2004). Local actions of hormone levels are also pertinent to the risk, with higher ovarian levels of both testosterone and androstenedione being related to an increasing degree of ovarian stromal hyperplasia in endometrioid endometrial cancer (Jongen *et al*., 2003).

Endometrial cancer and progesterone

The studies pertinent to this issue deal with the evaluation of the impact of menopausal hormone replacement therapy. A very large recent prospective study, the Million Women study, evaluated if women taking menopausal replacement therapy were at increased risk of endometrial cancer. Risk compared to never users with last users of continuous combined preparations show a reduction of the cancer risk with an RR of 0.71. In contrast, the risk was increased for oestrogen only at 1.45, and not significantly altered for last use of cyclic combined preparations at 1.05. These results were interpreted as being an indication that progestagens counteract the effects of oestrogens on the endometrium, the protective effect being proportional to the number of days every month on which they were added to the oestrogen (Beral *et al*., 2005). An increased COX-2 expression in hyperplasia may represent an early step in the carcinogenesis process (Orejuela *et al*., 2005). The Women's Health Initiative randomised trial confirmed that the risk of endometrial cancer is reduced with a Hazard Ratio (HR) of 0.81 among women who were allocated to the conjugated equine oestrogen plus medroxyprogesterone acetate group. However, compared to placebo, more women in that group required endometrial biopsies to assess vaginal bleeding. These data provide additional support for the continuous use of combined hormone preparations (Anderson *et al*., 2003). Two large case-control studies previously conducted among postmenopausal women in Los Angeles, showed that continuous combined oestrogen-progestin

replacement therapies are not associated with any increased risk of endometrial cancer. Sequential oestrogen-progestin therapies, with the progestin being given for at least 10 days per month, also did not increase risk. In contrast, sequential oestrogen-progestin therapy with the progestin being given for 7 days per month, did increase the endometrial cancer risk with only a relatively slight reduction in risk compared to the oestrogen only hormone replacement therapy effectively proportional to the reduction in the number of days of unopposed oestrogen (Pike and Ross, 2000). Similar results were also found in the Million Women Health Study, in which progestagens were found to counteract the adverse effect of oestrogens on the endometrium, the effect being greater the more days every month they are added to the oestrogen, and the more obese the women are (Beral *et al*., 2005).

In addition to the reduction in risk of occurrence seen for progesterone, endogenous progesterone also plays a role in the control of the tumour's proliferation activity and therefore in the prognosis of endometrial carcinoma (Boman *et al*., 1993). Furthermore it has been found that progestins activate vascular endothelial growth factor gene transcription in endometrial adenocarcinoma cells (Mueller *et al*., 2003).

In conclusion, evidence suggests that progestins, when added to oestrogens lead to an increased risk of breast cancer, whereas the same combination is associated with a reduced risk of endometrial cancer.

I-1.3. Ovarian cancer

Ovarian cancer is allocated to the group of hormone-dependant[11] cancers. Several histological types of ovarian cancer exist and their degree of hormone-sensitivity varies. The influence of hormones is mainly seen in ovarian adenocarcinoma, rather than in embryonal tumours and it has been hypothesized that the ovarian synthesis of sex steroids rather than their circulating levels are etiologically important. This assumption is based on the current evidence that elevated androgen and oestrogen levels, and decreased progesterone levels contribute to the pathogenesis of ovarian cancer, whilst at the same time a direct effect of gonadotrophins cannot be excluded. The observed results of an increased risk with ovarian androgens in premenopausal women, the lack of association with adrenal androgens, and the relatively weak association with obesity, hormonal replacement therapy and endogenous hormones after menopause are in favour of the hypothesis (Lukanova and Kaaks, 2005).

[11] Many studies have addressed the correlation beween oestrogen levels and cancer of the ovaries. These studies will not be discussed as oestradiol is not included in the mandate and Terms of Reference of this opinion.

Ovarian cancer and testosterone

Few studies addressed the ovarian cancer risk as a function of circulating levels of sex steroids. In a case-control study nested within 3 cohorts in New York, Umea and Milan, no clear association was observed between ovarian cancer risk in premenopausal women and any of the hormones under study, in particular not with testosterone, but with possible exception of androstenedione (Lukanova *et al*., 2003). Previously it had been concluded already that the measurement of testosterone was not a cost-effective method for the screening for the presence of androgen-secreting neoplasms of ovarian or adrenal origin (Waggoner *et al*., 1999).

The role of early exposure including *in utero* exposure still needs to be investigated. Polycystic ovarian syndrome patients have high serum androgen levels when pregnant, and these levels may lead to exposure of the foetus (Sir-Patermann *et al*., 2002).

An effect of testosterone on prognosis of ovarian cancer has hardly been investigated but seems unlikely. A clinical study concluded that the measurement of serum progesterone, 17β-oestradiol and testosterone was not helpful in detecting disease relapses (Marinaccio *et al*., 2000). *In vitro*, reduced survival of ovarian tumour cells has been described for lowered levels of testosterone (Taube *et al*., 2002, 2003).

Ovarian cancer and progesterone

Consensus exists about the protective effect of oral contraceptives against ovarian cancer (IARC, in press). Most of the recent studies deal however with hormone replacement therapy. In the Norwegian Women and Cancer study, no significant increase in the risk of ovarian cancer was found (Bakken *et al*., 2004). In the Women's Health Initiative randomised trial, the HR for invasive ovarian cancer was 1.58 for the equine oestrogens-medroxyprogesterone acetate combination (Anderson *et al*., 2003).

Mechanistic studies refer to the fact that progesterone inhibits the cell cycle and replication, presumably via down regulation of the cdkl/cyclin B complex (Zhou *et al*., 2002). Moreover, two functionally relevant polymorphisms in the human progesterone receptor gene (+331 G/A and progins) appear to be associated with risk for ovarian carcinoma, particularly among women under the age of 51 years (Romano *et al*., 2006). Genetic variation in the progesterone receptor gene has also been implicated in the risk for ovarian cancer (Terry *et al*., 2005) and the V660L germline variation in the progesterone receptor gene increases transcriptional activity and thus may modify ovarian cancer risk (Agoulnik *et al*., 2004).

In conclusion, despite the fact that ovarian cancers have been allocated to the group of hormone dependent cancer, data are too limited as yet to define the contribution of individual hormones (and/or combinations) to cancer risk.

I-2. Hormone-dependent cancers in males

I-2.1. Prostate cancer

The development and function of the prostate is dependent on androgens. It has been known since the 1940s that androgen depression by surgical castration, or more recently with medical treatment, has a favourable effect on the progression of the cancer. This observation led to the hypothesis that the rate of occurrence of prostate cancer could depend on levels of endogenous androgens and other hormones, and on levels of the same hormones as influenced by exogenous exposures (Grönberg, 2003; Marks, 2004; Morgentaler, 2006; Morales, 2006).

A number of studies have assessed the incidence of prostate cancer in relation to hormone measurements in biological samples obtained at a time prior to the diagnosis of cancer, using a nested case-control design.

In an early study, Eaton *et al.* (1999) analysed eight nested case-control studies, published before 1998, which reported analyses of prostate cancer incidence in relation to testosterone, non-SHBG bound testosterone, dihydrotestosterone, androstanediol glucuronide, androstenedione, dehydroepiandrosterone sulphate, sex hormone binding globulin, estrone, oestradiol, luteinising hormone and prolactin. The number of individual analyses varied between 114 cases and 114 controls and 817 cases and 2107 controls. No statistically significant association was found between any of the measured hormone level and prostate cancer incidence. Only for one of the hormones, androstanediol glucuronide, all four available studies found higher concentrations in cases of prostate cancer than in controls, but the statistical evaluation of the overall results showed only a weak significance.

Stattin *et al.* (2004) analysed a study on the basis of three cohorts in Finland, Norway and Sweden with a total of 708 cases and 2242 controls. For total testosterone and free testosterone there was a borderline statistically significant correlation between a decreasing incidence of prostate cancer and increasing levels of total testosterone and free testosterone. There was no association with sex hormone binding globulin.

Chen *et al.* (2003) reported a nested case-control study of 300 cases and 300 controls based on the Carotene and Retinol Efficacy Trial. Results were largely consistent with the studies described above. This study suggested a weak association with 3-alpha androstanediol glucuronide and a decreasing incidence of prostate cancer with increasing levels of free oestradiol.

Parsons *et al.* (2005) studied a cohort of 794 men with serial measurements of testosterone, and used a Cox regression model to quantify predictors of prostate cancer occurrence (n = 114). This analysis suggested a positive association between testosterone concentration and prostate cancer incidence.

Platz *et al.* (2005) reported a study of 460 prostate cancer cases and 460 controls from the Health Professionals' Follow-up Study. Overall this analysis did not suggest any association between testosterone and prostate cancer. However, when the analysis was stratified according to the Gleason score, testosterone levels were associated positively with low-grade disease and negatively with high-grade prostate cancer.

Severi *et al.* (2006) tested the hypothesis, suggested by Platz *et al.* 2005, that relatively high levels of testosterone protected against aggressive (high-grade) prostate cancer, using a study of 524 cases and 1859 controls from The Melbourne Collaborative Cohort Study. The hazard ratio for aggressive prostate cancer was 0.55 for a doubling of the concentration of testosterone, and 0.51 for androstanediol and 0.63 for DHEA. These differences were statistically significant and the authors concluded that high levels of testosterone and adrenal androgens are associated with a reduced risk of aggressive prostate cancer but not with non-aggressive cancer.

The most recent study by Tsai *et al.* (2006) conducted within the Child Health and Development Studies evaluated sex steroid hormones in young manhood in relation to subsequent prostate cancer in African-Americans and Caucasians. 119 African-American and 206 Caucasian cases were matched to 2:1 controls. The only significant association to emerge was an increased risk at 3.01 for the fourth to the first quartile of the ratio of total testosterone to total oestradiol only found for Caucasian men.

Prostate cancer incidence subsequent to manipulation of hormone levels or hormonal therapy

The growth of prostate cancer is androgen sensitive. The majority of patients with symptomatic disease respond to hormonal therapy, but the tumour eventually develops resistance and becomes androgen independent (Suzuki *et al.,* 2003; Härkönen *et al.,* 2005; Crawford, 2004). Treatment modalities include oestrogen, LH releasing hormone agonists, anti-androgens, gonadotropin releasing hormone antagonists, or intermittent androgen deprivation (or even castration).

In addition, a number of substances that modulate hormone levels have been introduced into therapy for various purposes. For example, Finasteride is used in the treatment of urinary obstructions due to benign prostatic hyperplasia. It inhibits the conversion of testosterone to its more potent metabolite dihydrotestosterone, which is active in the prostate. Thompson *et al.* (2003) reported a randomised trial of 18,882 men assigned either to Finasteride treatment or placebo. At the end of follow-up of this randomised trial the cumulative prostate cancer incidence was 18.4 % in the Finasateride group, versus 24.4 % in the placebo group. These findings suggest that the intervention with Finasteride had been effective in preventing the prevalence of prostate cancer. However, when the analysis was restricted to prostate cancer with a Gleason score of 7 or higher, the cumulative incidences were 6.4 % and 5.1 %, respectively, suggesting that Finasteride treatment increased the risk of occurrence of these high-grade cancers with a poor prognosis. The interpretation of these results is not straightforward and subject to an on-going debate in the

literature (Lotan *et al.,* 2005). It is noted that the incidence of prostate cancer in both study groups was inflated due to the sampling of biopsies and histopathological examination of tissue from all men in the study.

Comparable studies have been conducted with Dutasteride, an inhibitor of both iso-forms of 5-α-reductase that catalyse the conversion of testosterone into dihydrotestosterone even more effectively than Finasteride. Andriole *et al.* (2004) reported a study with 4,325 men randomised to Dutasteride or placebo treatment and followed for two years. The study was not specifically designed to quantify an effect on prostate cancer incidence, but the protocol recorded the occurrence of prostate cancer as adverse event. The cumulative incidence of prostate cancer was 1.2 % in the Dutasteride group and 2.5 % in the placebo group ($p = 0.002$). Data were too sparse for a quantification of the incidence of high-grade cancers.

Marks *et al.* (2006) reported in a preliminary communication a randomised trial of testosterone replacement therapy in 44 men with low testosterone levels and late onset hypogonadism. Testosterone replacement therapy increased testosterone levels to the normal range, but the prostate tissue levels of testosterone and dihydrotestosterone did not change significantly.

I-2.2. Testicular cancer

A recent systematic review assessed the epidemiological literature on prenatal oestrogen exposure and male reproductive disorders, including testicular cancer (Storgaard *et al.,* 2006). Proxy measures of oestrogen exposure included direct measurements, recorded intake of diethylstilbestrol, oral contraceptives and oestrogens, twin pregnancies and some environmental exposures. From the results of this analysis it was concluded that there was supportive, but not entirely consistent, evidence for the hypothesis that testicular cancer is associated with high prenatal oestrogen exposure.

The best-characterised oestrogen exposure is diethylstilbestrol and many cohorts of prenatally exposed men and women are being followed-up since the discovery that prenatal exposure to DES caused adenocarcinoma of the vagina in young women. For example, Strohsnitter *et al.,* (2001) followed 3,613 men of whom 1,709 had prenatal DES exposure. The rate ratio for testicular cancer was 3.1 (95 % confidence interval: 0.65 - 22.0) in comparison to non-exposed men in the cohort. The apparently higher incidence (based on 7 cases in exposed men, and 2 cases in unexposed men) was consistent with an effect of DES.

In conclusion, the evaluation of the risk for cancer in male individuals revealed that agents that block the conversion of testosterone to dihydrotestosterone reduce the overall incidence of prostate cancer, but increase in the incidence of high-grade prostate cancer.

Moreover, there is suggestive but not conclusive, evidence that high prenatal oestrogen exposure increases the incidence of testicular cancer in exposed males.

ANNEX II: IMPROVEMENTS IN THE ANALYSIS OF RESIDUES OF HORMONES IN ANIMAL TISSUES.

II-1. Improvement of sample preparation

Various methods for extraction and purification of androgens, oestrogens and progestagens have been developed for meat and meat products.

Initially, Stolker *et al.* (1998) presented a method involving supercritical fluid extraction for the determination of steroids in animal tissues. After addition of internal standards and sample pre-treatment, the analytes of interest were extracted from the matrix with unmodified supercritical CO_2 and trapped directly on an alumina sorbent placed in the extraction vessel (in-line trapping under supercritical conditions). After extraction, alkaline hydrolysis was performed and the analytes were derivatised prior to injection in GC-MS. Detection limits were 2 μg/kg for spiked samples.

Impens *et al.* (2002, 2003) developed a rapid and easy-to-perform method for the screening and confirmation of oestrogens, androgens, and gestagens in meat and kidney fat. After preliminary extraction, the steroid phase is defatted. Followed by a saponification step, the sample extract is purified by SPE. After derivatisation as trimethylsilylether derivatives, the final extract is analysed using GC-MS^2. Joos and Van Ryckeghem (1999) worked also on anabolic steroids found in kidney fat. They elaborated a procedure for the analysis of 36 anabolic steroids in this matrix. After a preparative HPLC, six fractions, containing the different steroids, were obtained. These fractions were then analysed, using LC-APCI-MS. When used in tandem mass spectrometry (MRM) mode, it is possible to obtain detection limits below 1 μg/kg for all steroids and below 0.1 μg/kg for most of them in spiked samples and incurred samples.

A rapid and economical method for the determination in meat of androgens, oestrogens, progestagens and corticosteroids was developed by Hartmann and Steinhart (1997). Extracted steroids are separated in a polar, a neutral and a phenolic fraction by C8-SPE followed by a liquid-liquid extraction of the phenolates. Each fraction is separately purified on normal phase SPE. The different steroid fractions are then sylilated and injected in the selected ion monitoring on a GC-MS (single quadrupole). With the same objectives, a method allowing detection of 23 anabolic steroids in the 5 - 100 ng/kg range (on real samples) has been elaborated (Marchand *et al.*, 2000). After extraction of the lyophilised meat, enzymatic hydrolysis was used for deconjugation. Indeed, different authors have demonstrated that some anabolic steroids present in meat were in conjugated forms (Maume *et al.*, 2001). Solid phase extraction on a polymeric stationary phase was then performed prior to hydrolysis of ester residues under alkaline conditions. Liquid-liquid partitioning was used to separate the analytes into two main categories: phenol containing molecules and Δ4-3-one containing molecules. Solid phase extraction on silica

columns was performed before applying a specific derivatisation for each compound sub-group and injection in GC-MS or GC-HRMS.

Shao *et al.* (2005) developed a method for simultaneous determination of residues of 11 illegal natural and synthetic steroids in foods of animal origin including porcine meat, porcine liver, porcine kidney, chicken and milk. Samples were concentrated using an Oasis HLB solid phase extraction cartridge, followed by dual silica and amino-propyl cartridges for cleanup. The analytes were quantified by liquid chromatography using a phenyl column coupled to an electrospray ionization tandem mass spectrometer (LC–ESI-MS/MS) operating in negative mode for oestrogens and in positive mode for androgens. The limits of detection for target analytes (spiked samples) were ranging from 1 to 120 ng/kg in different matrices.

Maume *et al.* (2001) improved the knowledge of the residue content in muscle, liver, kidney and fat of bovine origin treated or untreated with oestradiol as growth promoter. They developed a method to evaluate individually all the oestradiol free and conjugated forms present in edible tissues of animals including the measurement of the lipoidal (fatty acid esters) oestradiol at levels near the ng/kg range using gas chromatography-high resolution MS. The specific accumulation of lipoidal esters is a new area of investigation and could be of major importance in the exposure assessment according to their high potency (Paris *et al.*, 2001).

II-2. Improvement of derivatisation procedures

Very recently, Courant *et al.* (2006) employed a similar analytical strategy based on PFB-TMS derivatisation and GC-(NCI)-MS/MS for quantification of oestrogens in serum of children. Through a rigorous sample preparation procedure (LLE, SPE, Semi-preparative HPLC), they reached the ng/L level starting from a very restricted sample size (2 mL). Application of this methodology to meat samples would probably allow achieving the sub-ng/kg levels in food products of bovine origin.

II-3. Discrimination between endogenous and exogenous hormones

One of the most challenging tasks for the analyst in the field of chemical residue survey in food is the discrimination between endogenous production and exogenous administration.

Gas chromatography coupled to isotope ratio mass spectrometry (GC-C-IRMS) is definitively adapted to this differentiation. The principle of this method is based on the relative difference between the $^{13}C/^{12}C$ ratio of natural steroid compared with the $^{13}C/^{12}C$ ratio of the corresponding synthetic compound. The use of GC-C-IRMS allows the discrimitation between endogenous levels and exogenous administration of androgens (Ferchaud *et al.*, 1998; Ferchaud *et al.*, 2000; Prévost *et al.*, 2001, Hebestreit *et al.*, 2006) and oestrogens (Buisson *et al.*, 2005). This

methodology has not only been applied to urine samples but with success in various other matrices: kidney, liver and testicles (Prévost *et al.*, 2004). The future GC-C-IRMS technical improvements will undoubtedly result in the improvement of the sensitivity of the technique, making possible realistic and efficient natural steroid analysis in food producing animals.

Another way to distinguish treated from untreated animals is the study of the steroid metabolic profile. Maume *et al.* (2003) developed a very specific and sensitive method using GC-HR-MS for 17α-oestradiol, 17β-oestradiol, their corresponding hydrophilic phase II metabolites (glucurono-, glycosido-, and diconjugates forms) and lipoidal fatty acid esters. Muscle, liver, kidney and fat samples from control or single or multi-implanted steers have been assayed. The results demonstrated the possibility of differentiating untreated and implanted animals on the basis of differences in their metabolic profile. A multi-dimensional statistical approach confirmed the conclusions.

In conclusion, in the last few years, improvements in analytical techniques and in sample preparation have resulted in enhancement of detection limits in the analysis of meat products by GC-MS and LC-MS methods. Some laboratories are now able to detect and identify compounds (through MS^n or high resolution approaches) at the low level of 1 ng/kg. Improvements in mass analyser (accurate mass Time of Flight, Fourier Transform Ion Cyclotron Resonance and Orbitrap) will undoubtedly result in lowering the detection limits to the sub-ng/kg level as well as identify unknown compounds used illegally as growth promoting agents (Nielen *et al.*, 2006, 2007).

II-3.1. Synthetic hormones

All three compounds, i.e. zeranol, trenbolone and melengestrol acetate are known to form residues in animal tissues as outlined below.

II-3.1.1 Analysis of trenbolone residues

After 17β-oestradiol/trenbolone acetate implants according to authorised use, steer tissues had β-trenbolone concentrations in the 0.25 - 0.38 µg/kg range in muscle, liver and fat. The α-trenbolone residue level was even lower, except in liver (0.8 – 1.5 µg/kg). An implant of trenbolone acetate (without 17β-oestradiol) resulted in β-trenbolone and α-trenbolone concentrations 2 - 3 fold greater in liver, kidney and fat, but no greater in muscle than β-trenbolone in tissues of 17β-oestradiol/trenbolone acetate implanted steers (Henricks *et al.*, 2001).

Trenbolone is generally analysed as α- and β-trenbolone. An appropriate method for their analyses makes use of a fluorescence detector as an alternative. The detection limits for spiked

samples are estimated to be 0.2 and 1.0 µg/kg in bovine muscle and liver respectively (Yoshioka *et al.,* 2000).

A similar method, using UV detection yields detection limits for spiked samples of 0.2 µg/kg in muscle tissue and 0.6 µg/kg in liver tissue. Critical control points were identified in a pH adjustment step and an evaporation step. Analysis of incurred tissues (bovine liver and muscle) stored at -20°C for over 25 weeks did not identify any significant loss of residues (MacNeil *et al.,* 2003).

Even lower limits of quantification can be reached with UV detection for spiked α- and β-trenbolone: 1 µg/kg in muscle and 2 µg/kg in liver, after a stack-cartridge solid phase extraction (three columns) and multidimensional HPLC (Taguchi *et al.,* 2001).

An LC-MS method is capable of detecting trenbolone acetate, besides its two metabolites, in bovine muscle with a detection limit of 1 µg/kg in spiked samples (Tsai *et al.,* 2004).

II-3.1.2. Analysis of zeranol residues

Most of the recent analytical work addresses the fate of zearalenone, a mycotoxin that regularly contaminates feed commodities (see EFSA, 2004) and which can be converted into zeranol in ruminants. For example, analysis of muscle tissue (derived from animals that had been exposed to zearalenone) revealed relatively high amounts (up to 13.3 µg/kg) of zeranol, along with α-zearalenol (up to 14.5 µg/kg) and traces of zearalenone and taleranol. β-Zearalenol and zearalenone could not be identified in any of the investigated muscle samples, which at the same time indicate that the metabolite profile is a suitable marker to discriminate between the intentional use of zearanol and the un-intended exposure of animals to the mycotoxin zearalenone (Blokland *et al.*, 2006). The occurrence of the individual analytes was strikingly dependent on the type of muscle, since samples taken from the femoral region contained exclusively zeranol at low concentrations (0.5 – 2.1 µg/kg), while in samples from the back, zeranol was also accompanied by relatively high amounts of α-zearalenol (0.5 – 14.5 µg/kg) and occasionally traces of zeralenone and taleranol (0.5 – 1 µg/kg). Residue analysis of zeranol in bovine muscle tissue is similar to that of trenbolone and comprises liquid extraction, solid-phase cleanup, LC separation and MS detection. A method using negative mode electrospray ionization can reach detection limits of 0.5 µg/kg in spiked samples (Horie and Nakazawa, 2000).

Electrochemical detection allows quantification of zeranol at the 1 µg/kg level in spiked muscle tissues (Taguchi *et al.,* 2001). A method for zeranol residues in spiked bovine tissues does not use toxic chemicals, organic solvents and reagents in the sample preparation. Isolation is achieved by homogenization using a handheld ultrasonic homogeniser, followed by solid-phase extraction with an anion exchanger. The quantification limit is 40 µg/kg (Furusawa and Kishida, 2006).

Zeranol was included in a list of 18 mycotoxins and their metabolites, which can be identified and quantified in spiked bovine milk by liquid chromatography tandem mass spectrometry (Sorensen *et al.*, 2005). The detection capability, determined according to Commission Decision 2002/657/EC, was at 0.085 µg/L.

Zeranol was also included in a list of natural anabolic steroids and synthetic hormones which can be determined in spiked meat samples by freezing-lipid filtration, solid-phase extraction and GC-MS. A detection limit of 0.2 µg/kg was reported for zeranol after derivatisation into an OTMS derivative (Jungju *et al.*, 2005).

II-3.1.3. Analysis of melengestrol acetate

With regard to the detection of melengestrol acetate in bovine muscle tissue, a sensitive screening method, based on competitive microtitration plate enzyme immunoassay has been described. Samples were extracted with petroleum ether and purified with C18 cartridges. The detection limit for spiked muscle samples was 0.05 µg/kg (Hageleit *et al.,* 2001).

In conclusion, the introduction of high performance liquid chromatography coupled to mass spectrometry has permitted the detection of residues of trenbolone, zeranol, melengestrol and some of their metabolites at the sub-microgram per kg (µg/kg) range for liver and muscle tissues.