Genetic, environmental and life-style effects on androgen receptor function

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2012

GENETIC, ENVIRONMENTAL AND LIFE-STYLE EFFECTS ON ANDROGEN RECEPTOR FUNCTION

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2012

ISBN 978-91-87189-50-0
ISSN 1652-8220
Lund University, Faculty of Medicine Doctoral Dissertation Series 2012:87
Printed in Sweden by Media-Tryck, Lund University
Lund 2012

Till min familj

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Mol Hum Reprod. 2010; 16(3):153-157.

II. **Bjork C**, Nenonen, H, Giwercman A, Bergman A, Rylander L, and Giwercman Y. L. Persistent organic pollutants have dose and CAG repeat length dependent effects on androgen receptor activity in vitro.
Reprod Toxicol. 2011; 32(3):293-297.

III. **Bjork C** and Giwercman Y. L. The polyglutamine tract in the androgen receptor determines the effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on receptor activity in human prostate cells.
Under revision in Reprod Toxicol.

IV. Nenonen H., Skjærpe P.A, Lippolis G, Sajid Syed Khaja A, **Bjork C**, Bjartell A, Svartberg, J, Giwercman A, and Giwercman Y.L. Androgen receptor CAG length dependent amount of prostate specific antigen in serum and tissue.
Submitted manuscript.

V. **Bjork C**, Giwercman A, Brokken L, and Giwercman Y. L. Association between the CAG polymorphism and reproductive parameters and it's interaction with smoking in young Swedish men.
Manuscript under preparation.

ABBREVIATIONS

4,4'-DDT	2,2-*bis*-(4-chlorophenyl)-1,1,1-trichloroethane
4,4'-DDE	1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene
AF-1	activation function-1
AF-2	activation function-2
AGD	anogenital distance
AhR	aryl hydrocarbon receptor
AhRR	aryl hydrocarbon receptor repressor
AIS	androgen insensitivity syndrome
AMH	anti-Müllerian hormone
AR	androgen receptor
ARE	androgen-responsive element
ARNT	aryl hydrocarbon receptor nuclear translocator
B[a]P	benzo[a]pyrene
bHLH	basic helix-loop-helix
BPA	bisphenol A
CB-153	2,2',4,4',5,5'-hexachlorobiphenyl
CHO	Chinese hamster ovary
COS	African green monkey kidney cells
CUL4B	cullin 4B ubiquitin ligase complex
CYP1A1	cytochrome P450
DBD	DNA-binding domain
ddNTP	dideoxy nucleoside triphosphate
DES	diethylstribestrol
DHT	5α-dihydrotestosterone
E2	oestradiol
EDCs	endocrine disrupting chemicals
EPA	environmental protection agency
ER	oestrogen receptor
FSH	follicle-stimulating hormone
GR	glucocorticoid receptor
GnRH	hypothalamic gonadotropin releasing hormone
HAH	halogenated aromatic hydrocarbons
HPG	hypothalamic-pituitary-gonadal
HSP	heat-shock protein
LBD	ligand-binding domain
LH	luteinizing hormone
MR	mineralocorticoid receptor

NcoA4	nuclear receptor coactivator 4
NLS	nuclear localization signal
NTD	N-terminal transactivating domain
PAH	polycyclic aromatic hydrocarbon
PAS	Per-ARNT-Sim
PCa	prostate cancer
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PR	progesterone receptor
POPs	persistent organic pollutants
PSA	prostate specific antigen
qPCR	real-time quantitative PCR
SHBG	sex hormone-binding globulin
SMRT	silencing mediator for retinoid and thyroid hormone receptor
SOX	SRY-related HMG box
SP1	specificity protein 1
SRC-1	steroid receptor coactivator 1
SRD5A	5α-reductase
SRY	sex determining region Y
TAD	transactivation domain
TAU	transcription activating units
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDS	testicular dysgenesis syndrome
TGCC	testicular germ cell cancer
TRFI	time-resolved fluorescence imaging
WHO	World Health Organisation
XRE	xenobiotic response element

REVIEW OF THE LITERATURE

Androgens and androgen regulation

Androgens are steroid hormones that together with a functioning androgen receptor (AR) are found in varying amounts in virtually all human tissues. They are essential for sex differentiation and development in the foetus, establishment of sexual maturation at puberty including spermatogenesis and maintenance of male reproductive function thereafter. Androgens do not only act in reproductive tissues but also affect muscles, brain, kidney, skin, thyroid, fat and bone (Bennett *et al.*, 2010, Gelmann, 2002, Quigley *et al.*, 1995). It has recently been speculated that effects that are traditionally regarded as androgenic may actually be mediated by oestrogens acting via the oestrogen receptor (ER) (Carreau *et al.*, 2012).

The major circulating androgen is testosterone, which primarily is synthesised from cholesterol in the Leydig cells in the testis (Figure 1), whereas the adrenal glands are responsible for the production of a small amount. The irreversible reduction of testosterone to 5α-dihydrotestosterone (DHT) is catalysed by the enzyme 5α-reductase (SRD5A). DHT is considered as the most potent androgen because it has a slower ligand-receptor dissociation rate and is less susceptible to ligand-receptor degradation, compared to testosterone (Askew *et al.*, 2007). The androgen-bound AR is about 6 times more stable than the receptor without ligand (Kemppainen *et al.*, 1992). After dissociation from the ligand the receptor undergoes rapid degradation. Even though the affinity of DHT for AR is higher than for testosterone (Grino *et al.*, 1990), the level of DHT is only 2% of that of testosterone in the testes making testosterone the predominant intratesticular steroid (Jarow and Zirkin, 2005).

Androgens and other steroid hormones reach their target through the blood where they are bound to carrier proteins such as the sex hormone-binding globulin (SHBG) and albumin. As much as 98% of the testosterone is estimated to be bound, which makes only a small part biologically active. Both the total testosterone and bioactive androgen concentrations in intra-testicular fluid has been shown to be approximately 100-fold higher than the respective values in serum (Jarow and Zirkin, 2005). In the adult man, the mean total serum testosterone level is about 10-30 nmol/l of which roughly two-thirds are bioactive.

The hypothalamic-pituitary-gonadal hormone axis

In the adult man, the androgen production and spermatogenesis are regulated through a series of hormonal influences dependent on stimulation from the anterior

pituitary gonadotropins; luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Nieschlag and Behre, 2001). These gonadotropin hormones are secreted in response to the hypothalamic gonadotropin releasing hormone (GnRH). Since GnRH secretion is pulsatile, gonadotropin release also occurs in distinct peaks, which is most evident for LH since it has shorter half-time in circulation than FSH (about 20 min and two hours, respectively) (Nieschlag and Behre, 2001). The hypothalamic-pituitary-gonadal (HPG) axis is suggested to be activated by kisspeptin, a peptide hormone that can function as an essential gatekeeper to the onset of puberty (Hameed *et al.*, 2011). Kisspeptin can stimulate the release of GnRH through the kisspeptin receptor that is expressed by the GnRH neurons, which in turn stimulates gonadotropin release and subsequently LH and FSH. The main hormones involved in the adult male HPG-axis are demonstrated in Figure 1.

LH acts on the Leydig cells located between the seminiferous tubules in the testes, which responds by producing and releasing testosterone that together with FSH stimulates the maturation of spermatogonia into mature sperm in the Sertoli cells located in the seminiferous tubules (Nieschlag and Behre, 2001). Because there is no AR or FSH receptor expression in the germ cells, the effect of testosterone and FSH are most probably mediated by the Sertoli cells. This has been supported in cell specific knock-outs of the AR in mice during germ cell development (Verhoeven *et al.*, 2010).

Testosterone also circulates to exert a negative feedback control of LH secretion, directly or through aromatisation to oestradiol (E2), on GnRH pulse frequency from the hypothalamus. FSH acts directly on the Sertoli cells and is regulated by the peptide hormones inhibin that has an inhibitory effect and activin that has a stimulatory effect on FSH secretion from the pituitary. The peptides are produced in the Sertoli cells which also are responsible of providing a cell barrier to chemicals in plasma.

The androgen receptor

The AR belongs to the nuclear receptor superfamily together with other steroid receptors for oestrogens (ER), progestins (PR), glucocorticoids (GR), mineralocorticoids (MR) as well as thyroid receptors for retinoids (RAR/RXR), thyroids (TR), vitamin D (VDR), peroxisome proliferator activated receptor (PPAR) and aryl hydrocarbon receptor (AhR), together with orphan receptors that are still awaiting recognition of specific ligands (Janosek *et al.*, 2006). All are likely evolved from a common ancestral gene. These proteins are involved in regulation of a wide range of physiological functions in eukaryotic organisms

including cell growth and proliferation, differentiation or maintaining of homeostasis. They all share the same mode of action acting as transcription factors that is ligand-dependent signalling macromolecules modulating expression of various genes in a stimulatory or inhibitory manner (Janosek *et al.*, 2006).

The AR gene was originally cloned in 1988 (Chang *et al.*, 1988, Lubahn *et al.*, 1988, Tilley *et al.*, 1989) and is the only steroid receptor located on the X chromosome (Xq11-12), giving the karyotypically normal 46XY male a single copy of this essential gene. Apart from the full-length AR protein (110 kDa), an isoform lacking an intact amino-terminus (87 kDa) has been identified primarily in

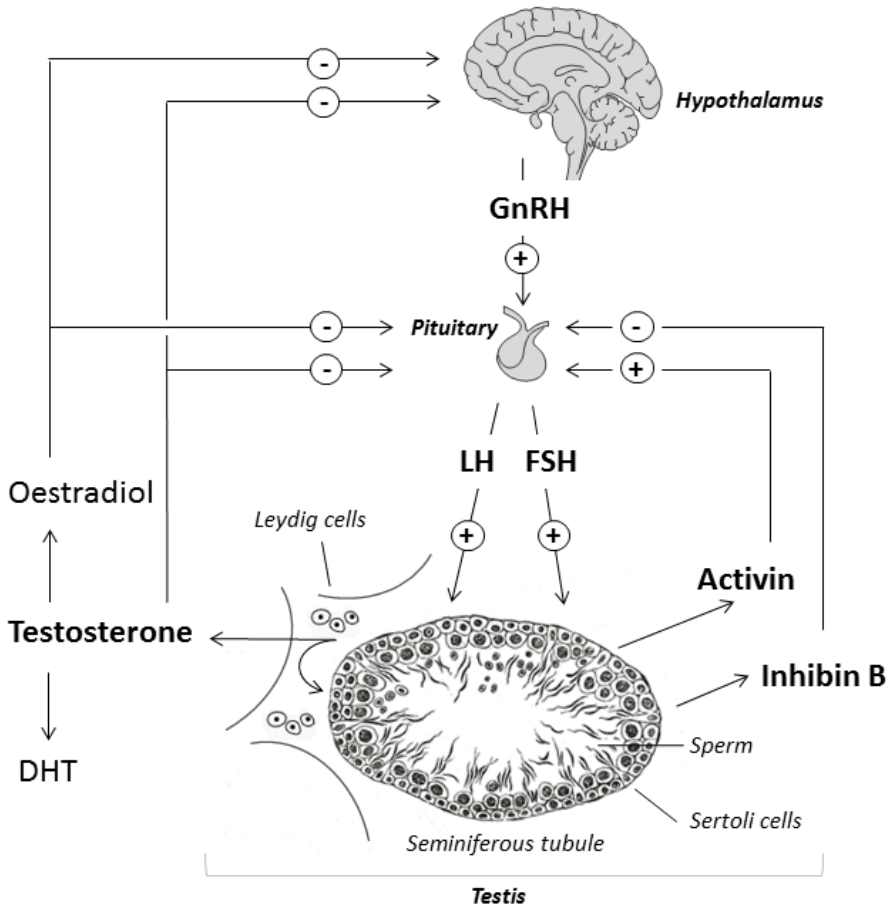

Figure 1. *Endocrine regulation of the hypothalamic-pituitary-gonadal (HPG) axis. GnRH: gonadotropin releasing hormone, LH: luteinizing hormone, FSH: follicle stimulation hormone, DHT: 5α-dihydrotestosterone. The seminiferous tubule is modified from the 20th edition of Gray's Anatomy of the Human Body.*

human genital skin fibroblasts (Wilson and McPhaul, 1994) and later also in heart, muscle, brain, uterus and prostate (Ahrens-Fath *et al.*, 2005). The shorter variant, called AR-A or AR45, is proposed to be due to translation initiation of AR protein at the internal methionine 188 residue of the full-length form (AR-B) (Wilson and McPhaul, 1994). The function of the A-isoform and other splice variants of the AR are still under investigation (Guo and Qiu, 2011). Since this thesis has its main focus on the role of the polyglutmine repeat that is located from amino acid 58 (Lubahn *et al.*, 1989) and downstream in the N-terminal domain, from this part only the full-length AR will be discussed.

The AR gene spans about 90 kb of DNA consisting of eight exons that codes for an approximately 2760 base pairs open reading frame within 10.6 kb mRNA (Gelmann, 2002) (www.ncbi.nlm.nih.gov/gene/367). The size of the protein varies depending on the length of the polymorphic repeats, but comprises approximately 919 amino acids (Lubahn *et al.*, 1989). Like the other members in the nuclear receptor family the AR includes four structurally and functionally distinct domains; a N-terminal transactivating domain (NTD), a DNA-binding domain (DBD), a small hinge region and a C-terminal ligand-binding domain (LBD) (Figure 2) (Lubahn *et al.*, 1988).

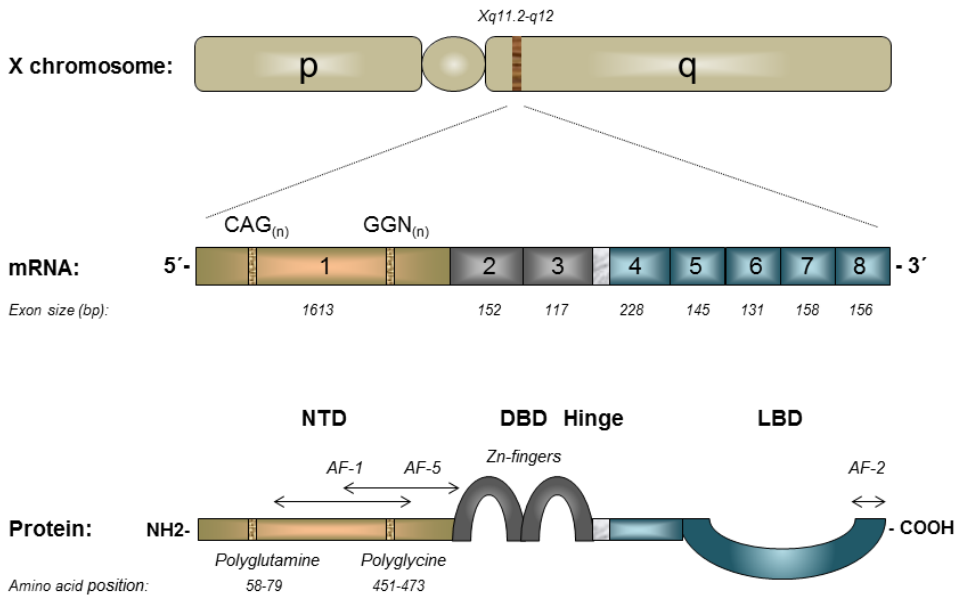

Figure 2. *The chromosomal location, mRNA organisation and protein domains of the AR. The gene consists of eight exons, where exon 1 encodes the transactivating domain with the two polymorphic CAG and GGN repeats. The amino acid positions for the CAG(n) and GGN(n) are based on 22 and 23, respectively.*

The N-terminal domain

The NTD is encoded by exon 1 and is the largest part of the protein (amino acids 1-537) (Lubahn *et al.*, 1989). This domain is the least homologous in sequence and variable in size between members of the steroid receptor family (Gelmann, 2002). It is also the part with the least evolutionary conservation, with only 20% amino acid identity with the rat. The NTD contains two polymorphic amino acid stretches, the glutamine and glycine stretches, that will be further discussed below. There are two overlapping areas responsible for the transactivation function in the NTD; activation function-1 (AF-1) (amino acids 142-485) and activation function-5 (AF-5) (amino acids 351-528) (Jenster *et al.*, 1995). These two regions encompass a number of peptide features such as microsatellite repeats, protein-protein interaction surfaces, phosphorylation- and sumoylation regulatory sites. They are sometimes also referred to as TAUs (transcription activating units) and the size and location of the active TAU in the human AR is dependent on the promoter context and presence or absence of the ligand-binding domain (Jenster *et al.*, 1995).

An interaction between the NTD and LTD during AR activation, also called N/C interaction, has been presented to be essential for complete AR activity (Jenster *et al.*, 1995). The first 30 amino acids in the NTD are important in interaction with the LBD activation function 2 (AF-2) domain, particularly the sequence located between amino acid 23 and 27 called ^{23}FQNLF^{27} and flanking regions (McEwan, 2004). The role of the N/C interaction is not clear, but the hypothesis is that conformational change after ligand binding eases the activation of the receptor by revealing the protein-DNA or protein-protein interaction surfaces that result in transactivation (Shen and Coetzee, 2005).

The DNA-binding domain

The DBD is encoded by the exons 2 and 3 (amino acids 538-627) (Lubahn *et al.*, 1989). Its main function is to recognise and bind to androgen-responsive gene promoters and enhancer regions. It is a central cysteine-rich region that has high degree of similarity with GR, PR, MR and ER and is 100 % identical to the rat AR. This group of steroid hormone receptors forms homodimers and all recognize palindromic sequences arranged as an inverted repeat and separated by three nucleotides. DBD consists of eight cysteine residues making up two motifs with a zinc ion bound in each, referred to as zinc-fingers (Freedman *et al.*, 1988). Within the first zinc-finger, encoded by exon 2, is a conserved motif called P-box that co-ordinates gene specific nucleotide contacts with the DNA major groove. The second zinc-finger comprises of exon 3 and contains another conserved motif (D-

box) which stabilises DNA receptor interaction by contact with the DNA phosphate backbone and mediates receptor homodimer.

The hinge region

The small hinge region (~50 amino acids) is encoded by the 5' section of exon 4 and is located between the DBD and LBD. It is a cluster of basic residues that contains the major part of the AR nuclear localization signal (NLS), which mediates the transfer of the AR from the cytoplasm to the nucleus (Bennett *et al.*, 2010, Quigley *et al.*, 1995).

The ligand-binding domain

The LBD spans over the 3' end of exon 4 and exons 5-8 (amino acids 678-919) (Lubahn et al., 1989) and comprises the steroid binding domain with specific, high affinity for androgens. The domain consists of 12 α-helixes that fold into an α-helical sandwich, as a ligand-binding pocket that allows for a docking of a number of coactivators proteins (Gelmann, 2002, McEwan, 2004). When helix 12 folds over the pocket to enclose the ligand and a hydrophobic cleft so called AF-2 is exposed on the LBD surface. The AF-2 then interacts with the NTD at specific so called FQNLF sequences (see above) (Gelmann, 2002). In the attendance of an antagonist, the helix 12 positions itself away from the pocket, thereby interfering with coactivators binding (Figure 3). Subsequently, a number of molecular chaperones such as the 90-kDa heat-shock protein 90 (HSP90) and other inhibitory proteins are released, the receptor is phosphorylated, it dimerises and the ligand-receptor complex is relocated to the nucleus to regulate target gene transcription. Following ligand addition, rapid and almost complete nuclear translocation is a common behaviour observed for almost all steroid hormone receptors (Griekspoor *et al.*, 2007).

The CAG polymorphism

The two stretches of glutamine and glycine in the AR N-terminal domain are specific for the AR, but are also present in the ARs of animals such as rats and primates (Choong *et al.*, 1998). The repeat lengths have been shown to decrease exponentially with evolutionary distance from humans and become polymorphic in the primates.

The most amino-terminal of the polymorphic repeats in the human AR is the $(CAG)_nCAA$ repeat encoding a stretch of glutamines, referred to as the CAG repeat (amino acids 58~79) (Lubahn *et al.*, 1988). The repeat length varies between individuals and populations of different ethnic origin, with African

American having on average fewer CAG repeats than Caucasians and Asians (Ackerman *et al.*, 2012, Edwards *et al.*, 1992, Kittles *et al.*, 2001). The mean CAG repeat lengths is in African-Caribbean 19.6 ± 3.2, Caucasians 21.9 ± 2.9, Hispanics 22.6 ± 3.1 and Thai 23.1 ± 3.3 (Ackerman *et al.*, 2012). In the general Swedish population the median CAG length is 22 amino acids, with variations from about 10 to 30 repetitions (Giwercman *et al.*, 1998). The CAG number distribution in Swedish men is shown in Figure 4. An abnormal expansion to >40 CAG causes spinal bulbar muscular atrophy, (SBMA), also known as Kennedy's disease (La Spada *et al.*, 1991). SBMA is a fatal neuromuscular disease, in which affected individuals are also present with oligospermia or azoospermia, testicular atrophy, and high plasma concentrations of LH, FSH and E2.

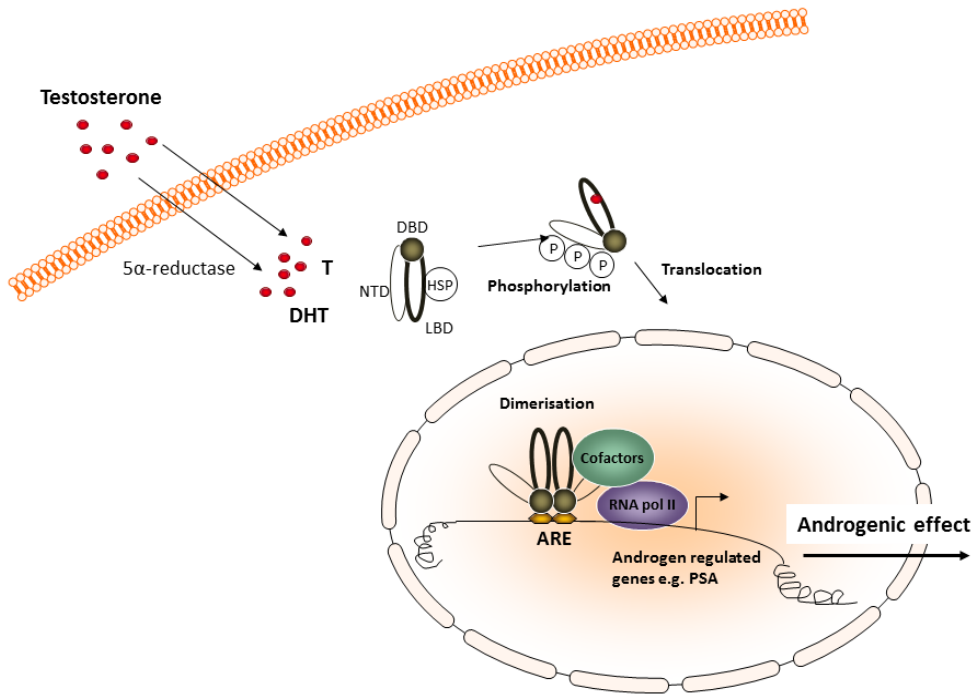

Figure 3. *General mechanism of androgen action in the cell. Inactivated androgen receptor (AR) is bound to heat-shock proteins (HSPs) in the cytoplasm. Upon ligand binding of androgens, the AR is phosphorylated and translocated to the nucleus where it dimerises and bind to androgen responsive elements (AREs) on the DNA of the target gene. Together with transcriptional coregulators and RNA polymerase II (RNA pol II) transcription of androgen dependent genes is induced. T:testosterone, DHT: 5α-dihydrotestosterone, NTD: N-terminal domain, DBD: DNA-binding domain, LBD: ligand-binding domain, P: Phosphorylation, PSA: Prostate specific antigen.*

Due to ethnic differences in CAG repeat length together with the fact that African Americans also have the highest incidence and mortality rate from prostate cancer (PCa), a connection between the CAG repeat and PCa has been extensively discussed. Some studies have found an association between CAG length and PCa risk (Akinloye *et al.*, 2011, Bennett *et al.*, 2002, Giovannucci *et al.*, 1997), age at diagnosis and response to endocrine therapy (Bratt *et al.*, 1999) and mortality (Giovannucci *et al.*, 1997), whereas others failed to find a link (Freedman *et al.*, 2005, Lange *et al.*, 2008, Salinas *et al.*, 2005, Tsujimoto *et al.*, 2004). A meta-analysis of 19 case-control studies showed that although the presence of shorter CAG repeats seemed to be modestly associated with PCa risk, the absolute difference was only on average 0.26 fewer repeats compared to controls (Zeegers *et al.*, 2004). Another meta-analysis furthered investigated this possible association and classified the studies by geographic areas as well as stratified the CAG repeat length in ≥20CAG vs. others, ≥22CAG vs. others and ≥23 vs. others, but no conclusive statistically significant associations were reported (Gu *et al.*, 2012). However, in the largest study to date on CAG repeat length and PCa risk that includes more than 6000 cases and controls, no association was found (Lindstrom *et al.*, 2010).

The association between CAG number and PCa has been further investigated in a mouse model of PCa where human AR CAG repeat variants of 12, 21 or 48CAG were introduced (Albertelli *et al.*, 2006). Animals with short and long CAG-

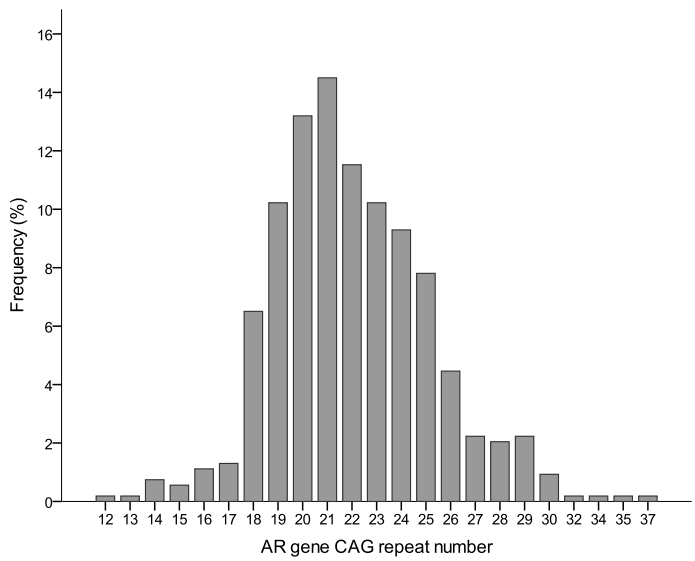

Figure 4. *The AR gene CAG length distribution in Swedish men from the general population (n=538).*

repeats had a longer PCa free survival, but when the disease was initiated, it had slower disease progression with a higher degree of tumour differentiation (Albertelli *et al.*, 2008, Albertelli *et al.*, 2006, Robins *et al.*, 2008). The short and long repeat lengths also had higher levels of expressed amount of AR protein than the 21CAG variant. Before PCa was induced in the mouse model, there were grossly normal in growth, behaviour, fertility and reproductive tract morphology although phenotypic analysis revealed that CAG number was inversely correlated to seminal vesicle weight and prostate lobe weights (Albertelli *et al.*, 2006, Simanainen *et al.*, 2011).

The CAG repeat have also been associated with reproductive function such as infertility and semen quality (Asatiani *et al.*, 2003, Davis-Dao *et al.*, 2007, Dowsing *et al.*, 1999, Giwercman *et al.*, 1998, Legius *et al.*, 1999, Mifsud *et al.*, 2001b, Mosaad *et al.*, 2012, Rajpert-De Meyts *et al.*, 2002b, Tse *et al.*, 2003, von Eckardstein *et al.*, 2001), reproductive hormones (Crabbe *et al.*, 2007, Fietz *et al.*, 2011, Huhtaniemi *et al.*, 2009), testicular cancer (Garolla *et al.*, 2005, Giwercman *et al.*, 2004b, Rajpert-De Meyts *et al.*, 2002a) and congenital malformations (Davis-Dao *et al.*, 2012, Parada-Bustamante *et al.*, 2012) giving inconclusive results. In this context one should bear in mind that in most studies, the associations have been analysed in a linear model, because of a general belief that the AR function diminishes with increasing CAG number. In a study on CAG repeat length and risk of subfertility with nearly 4000 subjects, CAG number was used in a stratified manner, and an association between short and long CAG numbers and increased odds of infertility was found (Nenonen *et al.*, 2011). A non-linear relationship between CAG number and reproductive outcome was also found in a novel study on the association between CAG number and cryptorchidism, that might be caused by a defected abnormal androgenic secretion and action during the development of external genitalia, where only shorter CAG repeats ($CAG \leq 19$ vs. $CAG \geq 20$) where associated with cryptorchidism (Davis-Dao *et al.*, 2012).

A number of experimental studies in cell lines such as African green monkey kidney cells (COS) and PCa PC-3 cells have been performed to elucidate the role of the CAG repeat on AR transactivation (Beilin *et al.*, 2000, Buchanan *et al.*, 2004, Chamberlain *et al.*, 1994, Ding *et al.*, 2004, Kazemi-Esfarjani *et al.*, 1995, Tut *et al.*, 1997). In one of the first studies comparing the transactivating capacity of vectors with 15, 20 or 31CAG repeats in COS-7 cells, a statistically significant difference was found between the short and long repeat variants, but no difference was found when comparing to 20CAG (Tut *et al.*, 1997). In another study, four different cell lines were transfected with similar CAG repeat lengths (15, 24 or 31CAG) (Beilin *et al.*, 2000). The statistical significant difference between the

short and long variants was only seen in COS-1 and PCa LnCap cells, whereas no differences were found in the PC-3 or breast cancer MCF-7 cells. The mechanism for the discrepancies in transactivating potential between 15 and 31CAG was further investigated in the COS-1 cells, but no dissimilarities in AR protein amount or AR-DNA binding were found. Decreased transactivating capacity was also found for the longer CAG variants above 35CAG (49 and 77) compared to 25CAG in a third study, whereas no difference was seen between 25 and 35CAG (Chamberlain *et al.*, 1994).

The CAG repeat has also been suggested to form a stable RNA stem-loop motif in which increasing number of repeats increases the length of the stem that in turn is capable of interacting with RNA-binding proteins (Yeap *et al.*, 2004). This means that the CAG repeat may function at both RNA and protein level; interacting with RNA-binding proteins at mRNA level that can modify both stability and translation and with AR coregulators at protein level to influence activity of AR as a nuclear receptor.

The GGN polymorphism

The second polymorphic repeat in the AR is located downstream from the CAG repeat (amino acids 449~472) and has consensus sequence $(GGT)_3GGG(GGT)_2$-$(GGC)_n$ (Lubahn *et al.*, 1988). The glycine stretch is referred to as GGN repeats, where the N represents cytosine, thymine or guanine.

The GGN repeat length also vary between individuals and populations of different ethnic origin, with African American having on average fewer GGN repeats than Caucasians and Asians (Ackerman *et al.*, 2012, Edwards *et al.*, 1992, Kittles *et al.*, 2001). As with the CAG repeat they also have the largest span of variation. In the Swedish population, GGN repeat length ranges from 10-27, with the most common alleles being 23 and 24, 52% and 32%, respectively (Lundin *et al.*, 2003).

It has been shown that a complete deletion of the GGN repeat segment reduces the trans-activating capacity of AR with approximately 30% (Gao *et al.*, 1996). AR with 23GGN has been shown *in vitro* to have the highest transcriptional activity compared to shorter and longer variants (10, 24 and 27) (Lundin *et al.*, 2007). Other *in vitro* studies have shown higher AR transcriptional activity with increasing GGN repeat length (Brockschmidt *et al.*, 2007, Werner *et al.*, 2006). This further demonstrates the promoter and cell-line specific outcomes of AR responsive genes and that are of importance for the functional outcome. The discrepancies between the studies might also be due to the CAG repeat variant used in the AR vectors.

The GGN repeat length has also been associated with reproductive function such as infertility and semen quality (Castro-Nallar *et al.*, 2010, Ferlin *et al.*, 2004, Lundin *et al.*, 2006), congenital malformations (Aschim *et al.*, 2004, Ferlin *et al.*, 2005, Parada-Bustamante *et al.*, 2012, Radpour *et al.*, 2007), testicular cancer (Garolla *et al.*, 2005, Vastermark *et al.*, 2011) and PCa (Akinloye *et al.*, 2011, Lange *et al.*, 2008, Platz *et al.*, 1998, Rodriguez-Gonzalez *et al.*, 2009, Vijayalakshmi *et al.*, 2006). Many of the studies show that genotypes other than 23GGN were associated with lower semen volume and congenital malformations, whereas others reveal that specific haplotypes of CAG/GGN can either increase or protect from infertility, cryptorchidism and PCa.

Transcription of the androgen receptor

The AR gene lacks a TATA and CAAT-boxes in the region immediately 5' to the start of the mRNA (Faber *et al.*, 1993). These motifs are generally recognised by the transcriptional machinery. Instead, as not uncommon with TATA-less genes, the region contains GC-rich sequences that bind the transcription factor SP1 (specificity protein 1). The gene is transcribed from at least two separate transcription initiation sites and the location of the androgen-regulated region is more than 2 kb downstream of the start site.

Apart from the well-characterised genomic pathway to regulate gene expression, a non-genomic pathway of AR signalling has been suggested (Bennett *et al.*, 2010). The nongenomic pathway acts in seconds to minutes which indicate a lack of transcription and translation from androgen-responsive genes. This action originates with numerous signalling molecules at the plasma membrane or in the cytoplasm, to trigger release of intracellular calcium and activation of protein kinases such as mitogen-activated protein kinase (MAPK), protein kinase B (PKB) and protein kinase C (PKC). The presence of a membrane-bound AR receptor has been postulated based on the detection of specific androgen binding to plasma membranes in different cell types (Heinlein and Chang, 2002). It is also possible that the non-genomic effects are mediated through SHBG or a c-sarcoma tyrosine kinase-AR complex. Although this reputed receptor type has not been purified or cloned, the identification of distinct membrane receptors for other steroid hormones such as se PR, suggests that a novel membrane receptor for androgens may also exist.

Androgen receptor regulation

Regulation of the AR is complex, being an age-time-, cell- and tissue-type dependent process. It can be regulated at all levels from gene transcription to protein activity and turnover rate. The AR is auto-regulated by androgens and both

up-regulation and down-regulation have been reported in different cell lines (McEwan, 2004). It can also be regulated by anti-androgens such as the DDT-metabolite; 1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene (4,4'-DDE) that in experimental studies has been shown to inhibit androgen binding and androgen induced transcriptional receptor activity (Kelce *et al.*, 1995). The overall effect of androgens at the mRNA level is down-regulation.

Through DNA microarrays and proteomic analyses a number of genes regulated by androgens coding for proteins involved in protein folding, trafficking and secretion, metabolism, cell-cycle regulation and signal transduction and cytoskeleton have been identified (McEwan, 2004). Examples of androgen regulated genes are prostate specific antigen (PSA), probasin, keratinocyte growth factor (KGF) and anti-apoptotic factor p21 that are induced and the tumour-suppressing serpin, maspin is repressed by androgens. Common for all these genes is that they have one or more AR-binding sequences together with binding sites for inducible and tissue-specific transcription factors.

AR coregulators are defined as "proteins that are recruited by the AR and either enhance (i.e. coactivators) or reduce (i.e. corepressors) its transactivation, but they do not significantly alter the basal transcription rate and do not typically possess DNA-binding activity" (Heemers and Tindall, 2007). Instead they influence AR mediating activity through acting at the target gene promoter region facilitating DNA occupancy, chromatin remodelling and/or recruitment of general transcription factors associated with RNA polymerase II. The coregulators can also assure the capability of the AR to enhance gene transcription directly through modulation of the proper folding of the AR and to ensure its stability or correct subcellular localization. Over 300 AR coregulators and interacting proteins have been reported (Gottlieb *et al.*, 2012) (http://androgendb.mcgill.ca/). The first to be isolated was SRC-1 (steroid receptor coactivator 1) in 1995 (Bennett *et al.*, 2010). SRC-1, together with TIF-1 (transcriptional intermediary factor 1) and GRIP1 (glutamate receptor-interacting protein 1) belongs to the p160 family that is a group of coactivators with a similar structural organization. They are characterised by three LxxLL (leucine-two other amino acids-leucine-leucine) hydrophobic motifs in the centre of the peptide sequence and a C-terminal glutamine rich region which are both used in nuclear receptor binding to the AF-2 region in the LBD to stabilise ligand-bound AR and thereby enhancing transcription (Bennett *et al.*, 2010, Shen and Coetzee, 2005).

Two well-known nuclear receptor corepressors are NCoR (nuclear receptor corepressors) and SMRT (silencing mediator for retinoid and thyroid hormone receptor). SMRT interacts with both the LBD and NTD and both repressors

constrain AR N/C interaction and compete with p160 coactivators (Bennett *et al.*, 2010). They do so by recruiting histone deacetylases (HDAC) which promote DNA packaging into nucleosomes, making the DNA inaccessible to transcription. The effect of SMRT has been shown to vary with the CAG repeat length in the AR in PCa cells (PC-3) (Buchanan *et al.*, 2011). It was also shown that the extent to which the CAG repeat influences AR activity is depending on the balance between corepressors and coactivator ratio. This may partly explain the diverging effect of the CAG polymorphism in different cell lines and its association with PCa risk.

AR regulations also occur through post-translational modifications such as phosphorylation, acetylation, methylation, sumoylation and ubiquitination at a total of 23 sites in the AR (Gioeli and Paschal, 2012).

The newly synthesized AR becomes phosphorylated within 10 minutes upon synthesis (Brinkmann *et al.*, 1999). This rapid modification is important for the acquisition of the hormone binding properties of the AR, although phosphorylation can occur both in the absence or presence of ligand (Gioeli and Paschal, 2012). Phosphorylation by kinases such as AKT, cyclin-dependent kinases and MAP-kinases is presumed to affect AR activity by increasing or decreasing protein interactions occurring proximal to the phosphosite. There is evidence for AR phosphorylation on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues and the majority of the phosphosites are located in the NTD. Phosphorylation has also been shown to affect recruitment to DNA, enhances and hormone binding.

The AR-mediated transcription concludes with ubiquitin-dependent destruction of AR by the proteasome (Gioeli and Paschal, 2012). Mass spectrometry has identified two sites for ubiquitination of the AR; Lys 845 and Lys 847. When a single ubiquitin attaches to an acceptor lysine, the chain that is formed via ubiquitin- ubiquitin linkages, acts as a degradation signal. There are three different ubiquitin E3 ligases known to modify AR and generate ubiquitin chains that target it for degradation; MDM2 (murine double minute 2), CHIP (carboxyl terminus of the HSP70-interacting protein) and RNF6 (ring finger protein 6). The ligand-activated AhR has been suggested to be a part of the E3 ubiquitin ligase that determines target specificity, integrated as a component of a novel cullin 4B ubiquitin ligase complex; $CUL4B^{AhR}$ (Ohtake *et al.*, 2007). Proteasomal degradation of AR in prostate has also been shown in an *in vitro* assay on rats neonatally exposed to abnormally high E2 concentrations, interrupting normal prostate development (Woodham *et al.*, 2003). These findings demonstrate an additional signalling pathway in which hormone or hormone-like chemicals can regulate AR function.

Male sex development and androgen action

The foundations for spermatogenesis are laid during fetal development and disturbances of events of this time may have subsequent impact on the quality of spermatogenesis and fertility in adulthood. Androgens play a central role is this process and are important in all phases of life.

Sex determination and differentiation

Fetal sex development is genetically determined by the presence of the sex chromosomes XY in normal males, where the Y chromosome is inherited by the father. The cascade of events can be divided into three stages; 1) the undifferentiated stage, when identical primitive structures develop into the XX and XY embryos, 2) gonadal differentiation into ovaries or testes and 3) the differentiation of internal and external genitalia, which is depending on the action of presence of testicular hormones into the male pathway or in their absence into the female pathway (Rey and Grinspon, 2011).

The SRY (sex determining region Y) gene is located on the Y chromosome and is the major initiator of male sex determination in almost all mammals (Kashimada and Koopman, 2010). SRY belongs to a family of SOX (SRY-related HMG box) genes that acts as transcription factors. SOX proteins have diverse roles in embryogenesis and in organ development, acting as cell differentiation switches. There are a number of genes implicated in regulating SRY expression that controls expressions of hormones and steroid proteins during embryonic development such as WT1 (Wilms tumour suppressor gene), SF1 (steroidogenic factor 1) and DAX-1 (duplicated in adrenal hypoplasia congenital on the X chromosome) (Rey and Grinspon, 2011).

Until around the 7th week of gestation, the embryos develop identically in the bi-potential gonad (genital ridges) before they begin to differentiate into either a testis or ovary (Rey and Grinspon, 2011). Gonadal cells become segregated into two compartments; testicular cord and interstitial tissue. Testicular cords are composed by Sertoli cells and germ cells, surrounded by basal membrane and peritubular cells. Sertoli cells produce Anti-Müllerian hormone (AMH) and inhibin B. AMH is responsible for the regression of Müllerian ducts in the embryo. Testosterone, secreted by Leydig cells, is responsible for Wolffian duct differentiation into internal male genitalia (epididymis, vas deferens, seminal vesicles, and ejaculatory ducts). Testosterone is converted to DHT that is essential for the formation of the male external genitalia (development of a urethral opening on the glans penis, growth of phallus and scrotum, urethra and prostate), which occurs between weeks 10 and 14.

Since the regulated masculinization process occurs within a defined period of development and is highly dependent of male hormones, susceptibility to endocrine disruption by exogenous chemical exposures is also confined to this period (Sharpe, 2010) and has been shown in the rat (Welsh *et al.*, 2007).

Androgen action in infancy, puberty and adulthood

Newborn boys have a testosterone peak the so called "mini puberty", when the testosterone levels rise to 10-15 nmol/l but then decline to pre-pubertal low levels at 6 months of age (Stukenborg *et al.*, 2010). The biological role for this peak is unknown, but it is speculated that it may add to the significant increase in Sertoli cell number that has been found to occur in the human testis during the first months of life.

Start of puberty is defined by an increase of the testicular volume to >3 ml, which is due to onset of spermatogenesis caused by Sertoli cell maturation and production of a large number of post meiotic cells. This is triggered by a significant increase of testosterone when the Leydig cells start to differentiate with a subsequent increase in inhibin B and gonadotropins and decrease in AMH expression (Rey *et al.*, 2009, Rogol, 2002). The sequence of the reawakening of the HPG-axis is that sleep-associated surges in gonadotropin, especially LH, gradually continue throughout the day, increasing the levels of testosterone and DHT. Following the increased gonadotropin and androgen levels, increasing growth rate and appearance of secondary sex characteristics develop such as muscles, bone, growth of phallus, facial body and pubic hair growth and spermatogenesis.

In the adult male, androgens are crucial for the maintenance of the reproductive parameters throughout life. More information on androgen action is found in the previous part on androgens and androgen regulation.

Androgen related pathological conditions

Androgen insensitivity syndrome

Already in 1988, when Lubahn and co-workers cloned the human AR, they concluded that the androgen insensitivity syndrome (AIS) likely results from a defect in the AR gene, but that definitive linkage between AR and the syndrome requires identification of a mutant AR gene from an affected individual (Lubahn *et al.*, 1988). This was confirmed in a later study from the same authors on 46, XY siblings with complete AIS, caused by the mutation Val866Met in exon 7, having normal female external genitalia with absence of uterus and fallopian tubes and

bilateral intraabdominally testes and blood testosterone in the normal range (Lubahn *et al.*, 1989). AIS patients have phenotypes that vary from completely female external genitalia to degrees of partial masculinisation (Jaaskelainen, 2012). The diagnosis of AIS is based on clinical findings, endocrine evaluation and family history. There are over 800 mutations in the AR reported to cause AIS (Gottlieb *et al.*, 2012) (http://androgendb.mcgill.ca/).

Male infertility

About 1 out of 7 European couples are infertile (World Health Organization, 1999). Male causes are found in half of the cases; leading to that approximately 7% of all men have fertility problems. Chromosomal abnormalities like Klinefelter's syndrome (47, XXY) and mixed gonadal dysgenesis syndrome may in a few cases be the cause of the problem as well as Y chromosome microdeletions and AR mutations. Mild AIS mutations are almost exclusively associated with some form of male infertility and the number of reported mutations have increased from 17 to 44 to the androgen receptor gene mutations database since 2004 (Gottlieb et al., 2012) (http://androgendb.mcgill.ca/). Furthermore, the majority of these mutations have been located in exon 1, suggesting that *AR* exon 1 mutations might be a cause of some cases of infertility. The CAG and GGN repeats have in some studies been associated with male infertility (Asatiani *et al.*, 2003, Castro-Nallar *et al.*, 2010, Davis-Dao *et al.*, 2007, Dowsing *et al.*, 1999, Ferlin *et al.*, 2004, Legius *et al.*, 1999, Mifsud *et al.*, 2001b, Mosaad *et al.*, 2012, Tse *et al.*, 2003, von Eckardstein *et al.*, 2001) whereas others could not find any association (Giwercman *et al.*, 1998, Rajpert-De Meyts *et al.*, 2002b). Other causes of infertility are cryptorchidism, mutations in genes involved in the HPG-axis, inflammations, urogenital infections and surgeries that can damage vascularization of the testis. About 50% of the infertility cases are of unknown aetiology. A number of endocrine disrupting compounds (EDCs) have been associated to impair male fertility; with the most adverse effects at high-exposure locations (see sections on EDCs and persistent organohalogen pollutants; POPs).

Testicular germ cell cancer

Testicular germ cell cancer (TGCC) is the most frequent diagnosed cancer in male adults between 20 and 40 years of age. Established risk factors for TGCC are cryptorchidism, contralateral testicular germ cell tumour, gonadal dysgenesis and familial testis cancer (Dieckmann and Pichlmeier, 2004). It has also been shown that sub-fertile men are at increased risk of developing TGCC (Dieckmann and Pichlmeier, 2004, Peng *et al.*, 2009).

The incidence varies considerably according to region and ethnicity, with Asian and African countries having low and Scandinavians the highest frequency. In northern Europe, a 3 to 4-fold increase during the past 30-40 years has been noted (Richiardi *et al.*, 2004). The incidence in the Nordic countries is highest in Denmark and Norway with 11 and 12 cases per 100,000 individuals, respectively (Engholm et al., 2010) (www.ancr.nu). The Swedish incidence is 6/100,000 and Finnish 5/100,000. Mortality rates have however decreased in all Nordic countries in 2008. These geographical differences have been proposed to be influenced by exposure to environmental factors in early life. It has been suggested that an excess of oestrogens *in utero* during embryogenesis may lead to that some of the primordial germ cells lose track of their normal development and become premalignat cells that are activated at puberty. Studies have shown an association between high maternal oestrogen and androgen levels in early pregnancy and increased risk of TGCC (Holl *et al.*, 2009) as well as increased levels of polychlorinated biphenyls (PCBs) in mothers of men with TGCC (Hardell *et al.*, 2003), strengthening this hypothesis. There have been several studies on polymorphisms in genes associated with TGCC such as those involved in the HPG-axis encoding the ER1 and 2 and the LH receptor (Brokken *et al.*, 2012) and the AR gene CAG and GGN polymorphisms (Garolla *et al.*, 2005, Giwercman *et al.*, 2004a, Vastermark *et al.*, 2011).

Prostate cancer

Prostate cancer (PCa) is the second most common cause of cancer and the sixth leading cause of cancer death among men worldwide in 2008 (Center *et al.*, 2012). Sweden is in the top 4 of 40 countries investigated with an incidence of 97/100,000 and mortality of 21/100,000. The incidence has increased in all Nordic countries since the 1950s, although the mortality is about the same (www.ancr.nu). This may be a result of the increased PSA testing (and screening) during the past two decades. Well-established risk factors are age, ethnicity and family history.

Since circulating androgens are essential for prostate development and also to some extent for PCa development and progression acting through the AR, this has been extensively studied in the aetiology of PCa. The accepted idea that AR activity always promotes cancer progression has recently been challenged when it was discovered that prostate AR can function both as a suppressor or promoter of PCa metastasis (Niu *et al.*, 2008). In prostate epithelial basal intermediate cells the AR acted as a tumour suppressor to suppress PCa metastasis, in epithelial luminal cells as a surviving factor, and in stromal cells as a proliferator to stimulate PCa progression.

Based on the importance of androgens in PCa, it has been assumed that high circulating levels of androgens increase PCa risk. Nonetheless, two large studies found no association between testosterone, E2 or DHT and PCa (Lindstrom *et al.*, 2010, Roddam *et al.*, 2008). There is also increasing evidence from epidemiology and animal studies that some EDCs may influence the development and progression of PCa. Men exposed to Agent Orange contaminated 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have been shown to have an increased incidence and more aggressive variant of PCa (Chamie *et al.*, 2008). However, this finding has been debated in editorial comments due to the self-reported methods used and has not been replicated (Pavuk *et al.*, 2006). Instead it has been suggested that service time in Southeast Asia, independent of Agent Orange exposure, appears to be associated with increased PCa risk. Additionally, no association between PCa and Agent Orange exposure was found in men referred for prostate biopsy (Zafar and Terris, 2001). It has been suggested that occupational EDC exposure through farming and factory-workers is associated with PCa (reviewed in (Van Maele-Fabry and Willems, 2003)), with the strongest exposure-response relationship for PCa mortality among almost 15,000 electrical capacitor manufacturing workers exposed to PCBs (Prince *et al.*, 2006). With the background that these compounds are lipophilic and lipid metabolism has been implicated in prostate carcinogenesis, a nested-case control study was performed in 776 cases and 1444 controls from the Agricultural Health study (Andreotti *et al.*, 2012). The authors found an interaction between the insecticide; terbufos and ALOXE3 (epidermis-type lipoxygenase 3) polymorphism and PCa risk, contributing to additional suggestion of gene-environment interaction in PCa.

Endocrine disruption and the male reproductive tract

A time-related deterioration of male reproductive health during the past few decades has been suggested (Carlsen *et al.*, 1992). The reports on abnormalities in reproductive organs and decreased semen quality, not only in humans (Toppari *et al.*, 1996) but also in animals (Edwards *et al.*, 2006, Gray *et al.*, 2001), have raised concern about an adverse trend regarding male reproduction.

Testicular dysgenesis syndrome

In 1992, a meta-analysis covering 61 papers demonstrated significantly lower sperm count from 1938 to 1990 in men from the general population (Carlsen *et al.*, 1992). This was followed by a report showing increased incidence of cryptorchidism (undescended testes), hypospadias (incomplete fusion of the urethral folds that form the penis), TGCC and hampered spermatogenesis in many industrialised countries the last 50 years (Toppari *et al.*, 1996). It has also been

shown that sub-fertile men and men with cryptorchidism and hypospadias are at increased risk of developing TGCC (Peng *et al.*, 2009, Schnack *et al.*, 2010).

It has been proposed that reduced semen quality, cryptorchidism, hypospadias and TGCC are symptoms of one underlying cause, the so called testicular dysgenesis syndrome (TDS) (Skakkebaek *et al.*, 2001), which probably arises already in fetal life due to Sertoli and Leydig cell dysfunction. The rapid pace of increase of reproductive disorders suggest environmental or life-style factors, rather than accumulation of genomic structural defects to be the most likely causes. This hypothesis was not only based on animal and *in vitro* studies, but also on the observation that sons of mothers treated with diethylstribestrol (DES) during pregnancy had with increased occurrences of testis abnormalities such as cryptorchidism and decreased semen quality compared to sons of mothers who received placebo (Toppari *et al.*, 1996).

Apart from the rise in testicular cancer incidence (McGlynn and Trabert, 2012), it has been debated whether the reduced semen quality and increased cryptorchidism and hypospadias exists at all and if there are common risk factors for the four TDS conditions (Akre and Richiardi, 2009, Fisch, 2008, Fisch *et al.*, 2010). Recently, a study on nearly 5000 Danish conscripts revealed that semen quality had not declined during the last 15 years, but on the other hand only one in four men had optimal sperm concentration and morphology from a fecundity perspective (Jorgensen *et al.*, 2012).

Endocrine disrupting compounds

Since a variety of chemicals have been found to disrupt the endocrine systems of laboratory animals as well as of certain fish and wildlife, resulting in developmental and reproductive problems, the US Environmental protection agency (EPA) set up a program to screen chemicals for their potential to produce effects similar to those by the female and male hormones and the thyroid system (Kavlock *et al.*, 1996). According to EPA, the definition of EDCs is "exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes". The first scientific statement where evidence was presented that EDCs have effects on male and female reproduction, breast development and cancer, PCa, neuroendocrinology, thyroid, metabolism and obesity, and cardiovascular endocrinology was published in 2009 (Diamanti-Kandarakis *et al.*, 2009).

Examples of EDCs that have been suggested to cause adverse effects on the reproductive system in humans and animals are insecticides (e.g. 2,2-*bis*-(4-

chlorophenyl)-1,1,1-trichloroethane; 4,4'-DDT), fungicides (e.g. vinclozolin), herbicides (e.g. atrazine, TCDD), nematocides (e.g. dibromochloropropane; DBCP), plastics (e.g. bisphenol A; BPA), plasticisers (e.g. phthalates), synthetic oestrogens (e.g. DES) and compounds present in cigarette smoke (e.g. polycyclic aromatic hydrocarbons; PAHs) (Diamanti-Kandarakis *et al.*, 2009, Luccio-Camelo and Prins, 2011, Sharpe, 2010). Many of these chemicals are ubiquitous in the environment, resulting in daily exposure through ingestion, inhalation and dermal contact and through occupational exposure in industrial or agricultural contexts (Schell *et al.*, 2010). Foetuses are exposed by direct transfer across the placenta and infants through lactation.

The mechanisms of these compounds involve a wide array of actions and pathways including the estrogenic, androgenic, thyroid and retinoid pathways where the compounds can act directly as agonists or antagonists, or indirectly via other nuclear receptors such as the AhR that can interact with the hormone receptors. The EDCs can also disrupt the pathway of steroidogenic enzymes, modify hormone receptor levels and alter the pattern of synthesis and metabolism of endogenous hormones (Diamanti-Kandarakis *et al.*, 2009).

A large number of EDCs like some PCBs, BPA, as well as phytoestrogens such as genistein, can compete with oestrogen binding to the ER (Kuiper *et al.*, 1998), whereas other chemicals like vinclozolin and 4,4'-DDE acts as anti-androgens by binding to the AR and block androgen-induced cellular responses (Kelce *et al.*, 1995, Roy *et al.*, 2004, Wong *et al.*, 1995). Vinclozolin has also generated epigenetic trans-generational effects in the germ cell line by altered DNA methylation, resulting in reduced spermatogenic capacity (Anway *et al.*, 2006). This observation has however not been able to replicate in any following studies. Other compounds that are proposed to act prenatally and cause TDS symptoms in rats are phthalates that can act as anti-androgens through decreased testosterone synthesis (van den Driesche et al., 2012). Rats exposed *in utero* with di(n-butyl) phthalate (DBP) displayed reduced distance from the anus to the base of the scrotum i.e. anogenital distance (AGD). The AGD has been proposed to be a sensitive measure of prenatal anti-androgen exposure. An association between phtalates and AGD was also found in boys exposed prenatally, suggesting that these compounds at environmental levels can adversely affect male reproductive development in humans (Swan et al., 2005).

It is also important to keep in mind that no individual is exposed to one chemical at the time, but rather to mixtures of toxic substances from many different sources. Consequently, it is of interest to investigate the behaviour of mixtures with possible additive (Birkhoj *et al.*, 2004, Hass *et al.*, 2012, Kjaerstad *et al.*, 2010)

synergistic (Christiansen *et al.*, 2009) and antagonizing effects (Rider *et al.*, 2008) on male reproductive development and function, acting either on the same receptor or through different mechanisms.

Persistent organic pollutants

Persistent organohalogen pollutants (POPs) are a group of compounds with long half-time which have low water solubility but high lipid solubility and therefore persist in the tissue and environment and accumulate in fatty tissues (Schell *et al.*, 2010). They are semi-volatile and can travel long distances in the atmosphere before being deposited (Schell *et al.*, 2010). Exposure to POPs has been suggested as potential cause of reproductive disturbances including reduced semen quality (Hauser *et al.*, 2003), testicular cancer incidence (Hardell *et al.*, 2003), PCa mortality (Prince *et al.*, 2006), imbalanced chromosome and birth sex ratio (del Rio Gomez *et al.*, 2002, Tiido *et al.*, 2006), reduced serum testosterone levels (Goncharov *et al.*, 2009) and impaired fetal growth (Govarts *et al.*, 2012).

Even though the production of the widespread PCBs and 4,4'-DDT has been banned for decades, the chemicals are still detected in the blood stream of animals and humans all over the world (Longnecker *et al.*, 1997). The persistent metabolite of 4,4'-DDT, 4,4'-DDE has shown to have negative effects on sperm motility and morphology, reported from studies in Mexico and South Africa where it still is used as an insecticide to treat malaria in some areas (http://www.atsdr.cdc.gov) (Aneck-Hahn *et al.*, 2007, De Jager *et al.*, 2006). PCBs are by-products of combustion and have also been used in carbonless copy paper, capacitors and transformers (Longnecker *et al.*, 1997). Exposure *in utero* to PCB-contaminated rice oil has shown to reduce sperm motility, increase abnormal sperm morphology, and lower sperm fertilizing potential in Taiwanese men after the so called Yusheng accident in 1979 (Guo *et al.*, 2000). Men exposed before 20 years of age also fathered less boys compared to age-matched and neighborhood-matched controls (del Rio Gomez *et al.*, 2002). The PCB congener, 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) is one of the most abundant of 209 congeners in biological extracts and correlates with the total lipid adjusted concentration of PCBs in plasma (Grimvall *et al.*, 1997, Longnecker *et al.*, 1997). CB-153 is therefore useful to elucidate possible associations between POP exposure and male reproductive parameters. In experimental studies, AR antagonistic properties was shown in PCa cells (Schrader and Cooke, 2003), whereas no effect was noted in Chinese hamster ovary (CHO) cells (Bonefeld-Jorgensen *et al.*, 2001). Some of the PCBs are classified as "dioxin-like" due to their structure similar to dioxins.

Dioxins are collectively referred to as halogenated aromatic hydrocarbons (HAHs), including polychlorinated dibenzo-p-dioxins, polychlorinated

dibenzofurans and biphenyls with molecular similarity (Schecter *et al.*, 2006). Dioxins are by-products of industrial and combustion processes and the main dioxin source for humans is diet, accounting for 90-98 % of the total exposure (Llobet *et al.*, 2003). The most toxic of the 75 dioxin congeners is 2,3,7,8- TCDD that became well known as a contaminant of Agent Orange herbicide use in the Vietnam war and has been associated with an increased incidence and more aggressive variant of PCa (Chamie *et al.*, 2008). These men were also more likely to be black and present with lower preoperative PSA concentrations in the circulation before radical prostatectomy (Shah *et al.*, 2009). After adjustment for race, these men still had increased risk of biochemical progression and shorter PSA doubling time after recurrence.

The highest TCDD exposure measured in a man, 50,000 times higher than that in the general population, was in Victor Yushchenko after he was poisoned in 2004 (Sorg *et al.*, 2009). Yushchenko and the most exposed individuals following the Seveso accident in Italy in 1976 when a trichlorophenol plant exploded and up to 30 kg of TCDD was released, developed chlorachne, that is a relatively insensitive and rare pathology following high-dose of chlorinated synthetic organic chemicals (Schecter *et al.*, 2006). Exposure of males with low concentrations of dioxin *in utero* and through breastfeeding in Seveso manifested an almost 50% lowered sperm concentration as well as 20% reduced sperm motility in adolescence (Mocarelli *et al.*, 2011). The adverse effects of TCDD after *in utero* and lactational exposure on the male reproductive system have also been shown in animal studies with outcomes such as delay in testicular descent, decreases in seminal vesicle and ventral prostate weights (Mably *et al.*, 1992, Simanainen *et al.*, 2004) and decreased sperm counts (Gray *et al.*, 1995). This further demonstrates the significance of the timing of POP exposure with respect to outcome.

Another class of dioxins is the non-halogenated PAHs. PAHs, such as benzo[a]pyrene (B[a]P) originate as by-products of incomplete combustion from multiple sources such as vehicle exhaust releases, cooking of food and also in cigarette smoke (Miller and Ramos, 2001). B[a]P, which can cross the placenta and has been declared as a human carcinogen, is also capable of tumour initiation, promotion and progression (Agency for Toxic Substances & Disease Registry, 1990). The adverse effects of B[a]P on reproductive function has been demonstrated in male mice exposed *in utero*, which resulted in decreased gonad size and impregnation of 35% fewer females than for control males (MacKenzie and Angevine, 1981). Moreover, in cultured rat testicular Sertoli cells, B[a]P exposure induced cytotoxicity through apoptosis (Raychoudhury and Kubinski, 2003) and the compound has also been shown to impair testicular steroidogenesis and epididymal function (Inyang *et al.*, 2003).

The potential carcinogenic damage has been suggested to be due to DNA adducts that can be formed when B[a]P is metabolised, which has been shown in a higher frequency in sperm DNA of smoking men compared to non-smokers (Zenzes *et al.*, 1999). The major part of the effects of both HAHs and PAHs are mediated through the AhR that is further described in the chapter on the AhR on page 36-39. Recent *in vitro* work in CHO cells has shown that PAHs can possess endocrine disruption activity, where some of the compounds were shown to decrease AR activity in the absence of the AhR (Vinggaard *et al.*, 2000). This finding was supported by a study in human LnCap prostate cells, which express low levels of AhR, where PSA protein level as a measure of AR activity was decreased and some of the compounds inhibited AR DNA binding to the androgen-responsive elements (AREs) (Kizu *et al.*, 2003).

Cigarette smoking

Despite the well-known health hazards caused by tobacco use, cigarette smoking is still highly prevalent in the general population. Cigarette smoke contains about 400 compounds belonging to a variety of chemical classes with different toxic properties such as B[a]P but also heavy metals like cadmium and lead, nicotine etc. (Dechanet *et al.*, 2011). Smoking has been associated with reduced sperm number, sperm concentration, semen volume and motility as well as decreased levels of FSH and increased levels of LH and testosterone (Ramlau-Hansen *et al.*, 2007a, Richthoff *et al.*, 2008). However, others found no associations on semen quality (Jensen *et al.*, 2004) or reproductive hormones (Halmenschlager *et al.*, 2009). Cigarette smoking has also been associated with lower sperm fertilizing potential (Sofikitis *et al.*, 1995) and to increased oxidative stress and DNA damage in the spermatozoa (Soares and Melo, 2008).

Smoking Indian men (n=178) were shown to have lower sperm motility, increased sperm morphological defects and decreased sperm DNA integrity, higher serum FSH and LH levels and decreased testosterone levels as well as longer CAG tracts compared to non-smoking men (n=126) (Mitra *et al.*, 2012). However, these men were recruited from the infertility clinic and infertile men might not be representative for the general population when it comes to susceptibility to environmental and life-style hazards. Additionally, no interaction studies on smoking and CAG number on the sperm parameters or hormones were performed.

Since the foundations for spermatogenesis are laid during fetal development and disturbances of events of this time may have subsequent impact on the quality of spermatogenesis in adulthood (Sharpe, 2010), studies have also been conducted on the effect of maternal smoking during pregnancy. In a study by *Storgaard et al.* from 2003 it was revealed that 43% of the Danish mothers were cigarette smokers

(Storgaard *et al.*, 2003). Sons to mothers who smoked more than 10 cigarettes per day during pregnancy had 48% lower sperm count. Other reports also demonstrate decreased semen volume, testicular size and sperm concentration in men exposed to cigarette smoke *in utero* (Jensen *et al.*, 2004, Ramlau-Hansen *et al.*, 2007b, Ravnborg *et al.*, 2011). The high prevalence of *it utero* exposure to cigarette smoke might explain the decreased semen quality in Danish men and geographical differences in the Nordic-Baltic area (Andersen *et al.*, 2000, Jorgensen *et al.*, 2002). A possible reason behind the decreased semen quality may be that prenatal exposure to maternal cigarette smoke reduces the number of germ and somatic cells in the human embryonic gonad (Mamsen *et al.*, 2010).

The Aryl hydrocarbon receptor

Structure and function

The aryl hydrocarbon receptor belongs to the nuclear receptor superfamily (Janosek *et al.*, 2006). These receptors share the same mode of action, mainly acting as transcription factors. The AhR is highly conserved in phylogeny from invertebrates to vertebrates and is the major regulator of drug metabolism activated by endogenous and exogenous ligands, as shown in AhR knock-out mice that are resistant to acute toxic, carcinogenic, and teratogenic effects of TCDD (Abel and Haarmann-Stemmann, 2010). Additionally, it is also essential for biological endpoints such as growth, apoptosis and differentiation and regulates normal development of reproductive tissues such as the seminal vesicles and the prostate (Lin *et al.*, 2002), the liver and the immune system (Gonzalez *et al.*, 1995), as well as lipid synthesis (Alexander *et al.*, 1998).

The AhR is located on chromosome 7p15 and consists of 11 exons (Abel and Haarmann-Stemmann, 2010). It encodes a protein of 848 amino acids and has a molecular mass of 96 kDa. The AhR, as well as its dimerization partner ARNT (Aryl hydrocarbon receptor nuclear translocator) and AhR repressor (AhRR), are characterized by two structural motifs, the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain. The N-terminal bHLH domain is required for DNA binding and protein dimerization and the PAS domain acts as a docking site for other PAS proteins (hetero- and/or homodimerisation) and the molecular HSP90. The gene also contains an N-terminal NLS and a nuclear export signal (NES) localized in the PAS domain. In the C-terminal domain there is a glutamine-rich transactivation domain (TAD) that interacts with several transcriptional coactivators and is therefore necessary for target gene activation.

The ability of the AhR to bind and be activated by a range of structurally divergent chemicals suggests a rather promiscuous ligand-binding site of the protein (Denison and Nagy, 2003). The most extensively characterized classes of AhR ligands are the environmental contaminants, such as the HAHs and PAHs and related compounds, although naturally occurring ligands such as flavonoids, iodole derivatives, curcumin and cartinoids also exists. The naturally occurring dietary ligands are plant products that are distributed in dietary vegetables, fruits and teas. Flavonoids have been measured in human blood in levels that are sufficient to inhibit or activate the AhR. Actually, the majorities of these compounds are known to inhibit TCDD- or B[a]P-stimulated AhR signalling and are therefore considered as chemopreventive agents (Abel and Haarmann-Stemmann, 2010).

The general mechanism of AhR transcriptional activation is demonstrated in Figure 5. Without ligand, the AhR resides in the cytoplasm, bound to two HSP90 molecules, the 23-kDa co-chaperone p23 and the immunophilin-like AhR interacting protein (AIP), that stabilises the protein and to devoid its uncontrolled nuclear translocation (Abel and Haarmann-Stemmann, 2010). Upon ligand binding, the receptor undergoes a conformational change, cofactors dissociate and the exposed NLS leads to shuttling to the nucleus. In the nucleus, AhR dimerises with its partner molecule ARNT via interaction of the HLH and PAS domains of both proteins. The AhR/ARNT heterodimer binds to regulatory sequences known as xenobiotic response elements (XREs) in the promoters of target genes, RNA polymerase II is recruited to the transcription machinery to induce gene expression.

The target genes encode enzymes, such as the xenobiotic metabolizing cytochrome P450 (CYP1A1), CYP1B1, and glutathione S-transferase (Mehta and Vezina, 2011), although the direct target genes of the AhR do not fully explain its physiological and toxicological effects. The induction of CYP1A1 (cytochrome P450) is the AhR dependent response that has been consistently observed in most species, and is therefore used as the model system to define the mechanism by which the AhR regulates gene expression (Denison and Nagy, 2003).

As for the AR, the AhR lacks a TATA-box in the promoter region and instead contain multiple GC boxes and SP1 transcription factor binding sites that are typical of promoters in house-keeping genes (Harper *et al.*, 2006). In addition, consensus sequences for six AP-1 and two AP-2 binding sites have been identified within the human AhR promoter (Abel and Haarmann-Stemmann, 2010). Two potential AhR/ARNT binding sites (AHRE-I) are clustered around the promoter between 0 and +750 nucleotides which indicates a possible autoregulation of AhR expression by its own ligands (Harper *et al.*, 2006). However, no functional

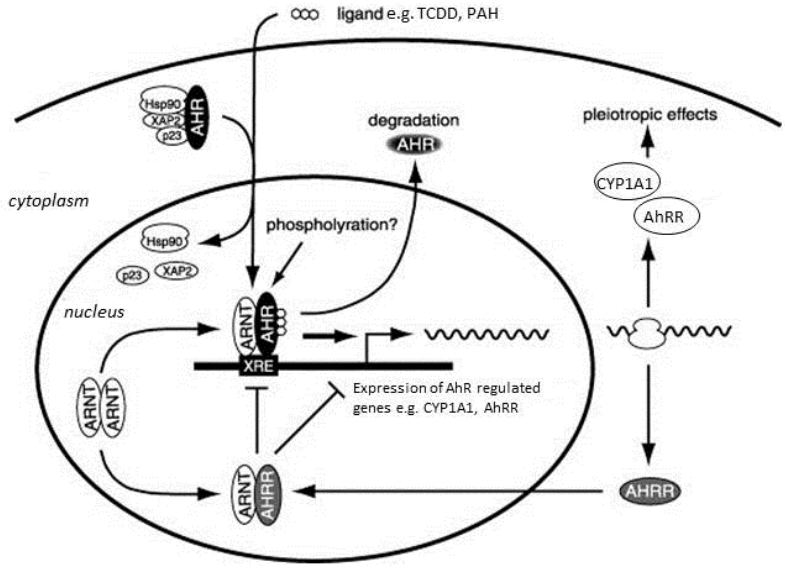

Figure 5. *Aryl hydrocarbon receptor (AhR) transcriptional activation with its dimerization partner aryl hydrocarbon receptor nuclear translocator (ARNT) and negative feedback regulation of aryl hydrocarbon receptor repressor (AhRR) (reproduced from Mimura and Fujii-Kuriyama (2003) with modifications).*

studies have been done to demonstrate that any of these sites are responsible for upregulation of AhR expression by AhR ligands. Another regulatory switch to balance AhR signalling, apart from proteasomal degradation is the AhRR (Abel and Haarmann-Stemmann, 2010). Because of its structural similarity with the AhR, the AhRR also dimerises with ARNT and bind to XREs. But, since the C-terminal part of AhRR lacks a TAD, DNA binding of the dimer leads to transcriptional repression.

Knocking out the AhR gene in a PCa mice model was shown to increase the PCa tumour incidence, suggesting that AhR has tumour suppressor properties (Fritz *et al.*, 2007). Mice have also been treated with an AhR modulator that, although not affecting prostate tumour incidence or size, inhibited tumour metastasis (Fritz *et al.*, 2009). However, some studies propose that AhR also plays a role in tumour initiation, since AhR regulates phase I enzymes that convert carcinogens to active genotoxins (Dietrich and Kaina, 2010). Recent evidence also suggests that activation of AhR might lead to deregulation of cell-cell contact, thereby inducing unbalanced proliferation, dedifferentiation and enhanced motility. This has led to speculations that low AhR signalling may protect against PCa, while high AhR signalling might promote it (Mehta and Vezina, 2011).

AR-AhR cross-talk

Experimental studies have demonstrated a cross-talk between AhR and steroid receptors such as ER and AR. In LnCap cells, TCDD blocked androgen-induced cell proliferation (Jana *et al.*, 1999, Morrow *et al.*, 2004). The mechanism behind this androgen-dependent disturbance of cell proliferation might be explained by a block of proteins required for the cell cycle to progress caused by TCDD (Barnes-Ellerbe *et al.*, 2004). TCDD has also been shown to decrease the AR protein levels (Morrow *et al.*, 2004), which may have been a result of the AhR ubiquitin ligase function. Ligand-bound AhR promotes the ubiquitination and proteasomal degradation of the AR by assembling the $CUL4B^{AhR}$ (Ohtake *et al.*, 2007, Ohtake *et al.*, 2009).

Co-treatment with TCDD has also been shown to decrease testosterone-dependent AR transcriptional activity in LnCap cells (Jana *et al.*, 1999, Morrow *et al.*, 2004). However, a study in PC-3 cells showed that TCDD/DHT did not have any impact on the DHT-induced AR activity (Kollara and Brown, 2010). The diverging effects between the cell-lines might be due to different promoters, varying concentration of ARNT as well as cell-specific transcription coregulators or varying metabolic degradation processes.

Except from the proposed ubiquitin ligase function of AhR, the cross-talk has been suggested to be caused by competition of co-factors at transcription level. One study found that NcoA4 (nuclear receptor coactivator 4, also known as ARA70) - facilitation of AhR activity was abolished by overexpression of AR, proposing a potential competition of AhR and AR for NcoA4 (Kollara and Brown, 2006). A second study showed that the AR coactivator FHL2 (four and a half LIM domain 2) level was found to alter the AhR modulation of AR activity (Kollara and Brown, 2010) . Another proposed mechanism for the interaction is that the AhR associates with the AR on androgen responsive promoters, such as the PSA promoter, as a transcriptional co-regulator (Kollara and Brown, 2010, Ohtake *et al.*, 2008).

It has also been shown that DHT significantly facilitated the complex formation between AR and PAH-bound AhR, which in turn suppressed transcription of AhR dependent genes (Sanada *et al.*, 2009). This demonstrates the bi-directional cross-talk between the receptors. The AR/AhR complex was proposed to have decreased binding affinity for the XRE which inhibit recruitment of coactivators and formation of the transcriptional machinery through steric hindrance.

Genetic predisposition of endocrine disruption on the male reproductive system

A possible target gene for a gene-environment interaction effect on reproductive function is the AR with its polymorphic repeats. It has been shown in a study on 680 men from Greenland (Inuit), Poland, Ukraine and Sweden where serum levels of 4,4'-DDE and the PCB congener, CB-153, were measured and correlated to semen parameters (Giwercman *et al.*, 2007). In general, no association between POP levels and sperm concentration was found. However, when the AR CAG genotype was taken into account, high CB-153 exposure was linked to 35% reduction in sperm concentration in subjects with <20CAG. Even though these men were proven fertile, the study indicates that men with certain AR genotypes might be more susceptible to the adverse effects of POPs.

It has also been speculated whether Inuit men that are highly exposed to POPs and have one of the lowest PCa incidences in the world (Friborg *et al.*, 2004), are somehow genetically protected. A study comparing low-risk Inuit and high-risk Scandinavian men demonstrated that high-activity polymorphisms in the SRD5A2 occurred less frequently in Inuit men (Giwercman *et al.*, 2008). Furthermore, they had longer median CAG repeats, suggesting a genetically predisposition to lower activity in testosterone to DHT turnover and to lower AR activity that might protect against PCa. These men also had higher frequency of the 23GGN allele that has been proposed to be protective against hypospadias (Giwercman *et al.*, 2006).

A number of epidemiological and experimental studies have reported the estrogenic, anti-estrogenic, dioxin-like and androgen-competing properties of EDCs. At the start of this PhD project, apart from one epidemiological study, it was not known whether certain CAG genotypes could have a protective genetic effect against environmental or life-style influences or if it could modify the effect of the pollutants acting through the AR or as an interaction with other steroid receptors such as the AhR.

AIMS

The overall aim was to investigate the role of the CAG polymorphism on AR activity and expression and whether it could modify the effect of environmental pollutants on AR function.

The specific aims were to:

- determine the effect of CAG repeat variants within normal range on AR activity and protein amount *in vitro*;
- investigate any potential modifying effects of CAG repeat length after exposure to CB-153 and 4,4-DDE on AR function *in vitro*, as single compounds and in a mixture;
- study whether TCDD has a CAG length dependent effect on AR function in prostate cell lines;
- examine whether PSA concentration in serum and amount in non-malignant prostate tissue are CAG number dependent;
- analyse the possible association between CAG number and reproductive parameters utilising a unprejudiced spline regression model, a stratified model as well as a linear model. We also wanted to investigate CAG number as a modifier of smoking, current or *in utero* exposure, on the same reproductive outcomes in young Swedish men.

MATERIALS AND METHODS

Study populations

Swedish military conscripts (IV and V)

In 2000-2001, approximately 95% of all 18 years old Swedish males underwent a medical health examination prior to military service. Since only those with chronic diseases were excluded, these men closely represent the general population of young Swedish men. A total of 2225 men born between 1979 and 1982 and living <60km from Malmö where invited to participate in the study. Among these, 305 (14%) accepted. In order to minimise the possible effect of ethnic variation, and since the AR is located on the X chromosome, only men born and raised in Sweden, with Swedish mothers (n=224), were selected for this study. The median age at examination was 18 years (range 18-21 years).

In 2008-2010, only 25% of all Swedish men around 18 years old underwent medical health examination prior to military service due to savings in the military budget. All 1681 men who undertook the examination and lived <60km from Malmö as well as born and raised in Sweden, with Swedish mothers, were enrolled in a study to address the issue of the suggested time-related reduction in sperm counts in the Western world. Among these, 241 (14%) accepted to participate. To reach a number of about 300 men as in the first cohort, another sub-cohort of 73 men of the same age fulfilling the same criteria as the conscripts were recruited through advertisement in schools or as friends of participants. Altogether, the second cohort consisted of 314 men, with a median age of 18 years (17-20 years).

All subjects signed an informed consent according to protocols approved by the ethical review board of Lund University and were paid 500 SEK for their participation.

The men underwent an andrological physical examination that included assessment of length and weight for calculation of BMI (body mass index). Testicular size was measured by use of ultrasound and orchidometer. A blood sample was drawn between 9 and 11 a.m. for hormone and DNA analysis. The participants were also asked to deliver a semen sample and fill in a questionnaire prior to the examination. The same questionnaire was used for both cohorts containing questions about factors that might influence their reproductive function, such as current own smoking and mother's smoking during pregnancy.

Assessment of semen volume, sperm concentration and sperm motility was performed according to the World Health Organisation (WHO) guidelines from

1999. The men were asked to keep 42-72 h of abstinence but in each case the actual abstinence time was recorded. Sperm motility was divided into four categories according to the WHO recommendations, A, B, C and D, with A corresponding to rapid progressive motility, B to slow progressive motility, C to non-progressive motility and D to immotile spermatozoa. Analyses of the ejaculates were performed by laboratory assistants in the laboratory, which is a reference unit for the external quality control of the European Society of Reproduction and Embryology and the Nordic Association for Andrology (ESHRE-NAFA).

The levels of FSH, LH, testosterone, E2 and SHBG in serum that are routinely measured and serve as markers of the reproductive system, where assessed. The hormones for cohort A were measured on an automated fluorescence detection system at the routine clinical chemistry laboratory at Uppsala University Hospital. In cohort B, the hormones were analysed on an ElectroChemiLuminiscence Immunoassay at the routine clinical chemistry laboratory at Skåne University Hospital, Lund, whereas E2 was determined by an immunofluorometric method at the routine clinical chemistry laboratory at Skåne University Hospital, Malmö.

Norwegian elderly men (IV)

The Tromsø study is an ongoing general health survey that was performed for the fifth time in 2001. All men and women, 30 years or older, living in the city of Tromsø and who had participated in the second phase of the fourth Tromsø study in 1994/1995 or became 30, 30, 45, 60 or 75 years old during 2001, were invited to be a part of the study. Of these men, 172 were four years later invited to participate in a study on late-onset hypogonadism. At the time of inclusion in 2005, they were between 60 and 80 years old and who had no priori diagnosis of prostate disease. All men participated after given written informed consent according to protocols approved by the ethical review board at the Regional Committee for Medical and Health Research Ethics, North Norway.

At the follow-up visit blood was drawn in the non-fasting state between 8 and 11 a.m. and serum samples were analysed for testosterone and PSA. Serum testosterone and PSA were detected by electrochemical luminescence immunoassay (ECLIA) using an automated clinical chemistry analyser.

Molecular methods

Polymerase chain reaction (IV and V)

Polymerase chain reaction (PCR) is a method that allows amplification of short DNA sequences (usually 100 to 600 bases) within a longer double stranded DNA molecule. PCR amplification entails the use of a pair of primers that are short oligonucleotides, each of about 20 bases in length that determine the specificity of the PCR. The primers are complementary to the sequences flanking your genomic area of interest. The PCR consists of three steps governed by temperature; denaturation, annealing and extension. In short, the template DNA is heated up to 96°C and the complementary double strands are separated. At a lower temperature the primers bind and are extended by a thermostable DNA polymerase (annealing). Copying of the single stranded DNA adjacent to the primer binding site is initiated in a 5´-3´direction (extension) and includes incorporation of deoxynucleotides in the PCR mix. This procedure is repeated at several (often about 40) rounds in a logarithmic manner and the products are thereafter run on an agarose gel for detection.

In this work, PCR was used to amplify the AR CAG region in men. Primarily, genomic DNA was extracted from peripheral leucocytes. The AR gene CAG repeat length was amplified in a 50 µl PCR reaction containing approximately 100 ng DNA, 0.3 µM of each of the primers: forward, 5'-TTAGGGCTGGGAAGG-GTCTA-3' and reverse, 5'-TGGGGCCTCTACGATGGGCT-3', 1.5 mM $MgCl_2$, 200 µM of dATP, dCTP, dTTP and dCTP each, 45 mM KCl, 10 mM Tris ultrapure and 1 unit of Dynazyme DNA polymerase. Amplification was performed for 42 cycles in an Eppendorf Mastercycler with denaturation at 96ºC for 1 min, annealing at 61ºC for 45 seconds, extension step at 72ºC for 2 min, with an initial denaturation step at 96ºC for 3 min and a final extension step at 72ºC for 7 min. The PCR results were verified by direct sequencing.

DNA sequencing (IV and V)

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. The most prevalent method is with the use of dideoxy nucleotides, also called Sanger sequencing or chain termination method. It is based on the addition of ddNTPs (dideoxy nucleoside triphosphates) to mixture with the four dNTPs used to synthesise DNA by extending the 3´end of the primer by the DNA polymerase. These extra nucleotides are synthetic and lack the -OH at the 3′ carbon atom. Therefore, incorporation of a ddNTP will stop the chain elongation by inhibiting the binding of an additional nucleotide to the next phosphodiester bond. If the ratio of dNTPs and ddNTPs is high enough, some strands will add

several hundred nucleotides before insertion of the ddNTP terminates the elongation. Each ddNTP contain a fluorescent or radioactive tag that is unique for each base, and therefore all four ddNTPs run in a single reaction instead of individually. The fragments are separated by length through gel electrophoresis or in a capillary gel electrophoresis system. The advantages with Sanger sequencing is that it gives an accurate read-out of the sequence obtained, which is beneficial when studying the number of CAG nucleotides as in our case. This also makes it possible to detect potential mutations in the amplified sequence.

In this thesis, prior to the capillary gel electrophoresis, the PCR products were dissolved in highly de-ionised formamide, that it is a polar solvent capable of keeping DNA denatured but still do not compete with the DNA during the electrokinetic injection into the capillaries in the sequencing gear. The samples were directly sequenced on an eight-capillary AB3130xl Genetic Analyser that is based on chain-termination along with the use of fluorescent ddNTPs.

Plasmid construction (I)

The most common AR GGN length in Caucasians of 23 was selected to generate expression plasmids in combination with 16CAG or 28CAG. A reference plasmid harbouring 23GGN and the median CAG length of 22 in Caucasians was already available in the research group. PCR was used for amplification of the 23GGN region and the reaction was run under the following conditions; 1 min denaturation at 96 ºC followed by a 1 min annealing at 56 ºC and 3 min extension at 72 ºC. The forward primer sequence was: 5´-CCAGAGTCGCGACTACTACAACTTTCC-3´ and for the reverse: 5´-CCAGAACACAGAGTGACTCTGCC-3´.

To contain the required GGN region of 241 bp, the PCR products were digested with Kpn1 and BstE11/Eco911. Two expression vectors (pCMV4) with the full length AR cDNA and the wanted CAG repeat length in combination with the unwanted GGN repeat were digested with the same enzymes as for the PCR product to exclude the GGN repeat. The opened plasmids without the GGN repeat were subsequently purified.

Ligation of the digested and purified PCR product with 23GGN into the opened pCMV4 expression vectors was performed, resulting in two vectors with 16CAG/23GGN and 28CAG/23GGN. The plasmids were transfected into Escherichia coli DH5α by electroporation, amplified and purified. As a last step, direct sequencing was performed to verify correct incorporation of the GGN fragment and the CAG sequence.

Transient transfection and reporter gene assay (I-IV)

The process of introducing nucleic acids into eukaryotic cells by non-viral methods is defined as transfection. This gene transfer technology is used to study gene function and protein expression in the context of a cell, which can be done by using various chemical, lipid or physical methods. Mammalian cells can be either transiently or stably transfected. Stable transfection integrates the foreign DNA into the cellular genome, whereas transiently transfected cells only express the genes in the foreign DNA for a short period of time, until the transfected DNA is degraded. The benefit with transient transfection is that provides great flexibility and speed for the early stages of investigational product development. Cells are typically harvested 24–72 hours post-transfection to analyse transient expression of transfected genes.

In study I, II and IV, COS-1 cells for transient transfection of AR expressing vectors were utilised. COS-1 cells do not contain any receptors for steroid hormones nor have endogenous steroid production and have previously been used in similar studies (Beilin *et al.*, 2000, Tut *et al.*, 1997). This cell-line is suitable for transient transfection because it contains a single integrated copy of the complete early region of the SV40 genome that expresses the viral T antigen, required for replication of viral T DNA. This makes it compatible with the expression vector, pCMV4, containing the SV40 origin of replication, which is recognised by T-antigen and causing the plasmid to be massively replicated.

In study III and IV, we used two human prostate cell lines; the benign prostate PNT1A cells and metastatic PCa PC-3 cells. None of the cell lines express AR protein (Mitchell *et al.*, 2000) and both express the AhR. PNT1A cells derive from a prostate of a 35 year old male at post mortem. The cell line was established by immortalisation of prostatic epithelial cells by transfection with a vector containing SV40 genome with a defective replication origin and therefore expressing the large T-antigen. These cells have previously been transfected for studying AR transcriptional activity and trafficking by analysing several actions of antiandrogens (Avances *et al.*, 2001). PC-3 cells are androgen-independent cells that derive from a bone metastasis of grade IV prostatic adenocarcinoma from a 62-year old Caucasian male. They have also been used in similar studies on AR-AhR cross-talk (Kollara and Brown, 2006, 2010).

The cell lines were incubated at 37°C with 5% CO_2 and kept in RPMI 1640 medium (prostate cells) and Dulbeccos' modified eagle medium (COS-1) containing phenol-red supplemented with 2 mM L-Glutamine, 10% fetal bovine serum and 0.02%.

Approximately 140.000-300.000 cells per well were seeded in 12-well plates and grown for 24 hours to reach 50-80% confluence. The cells were transiently co-transfected by the multi-component FuGENE 6 transfection reagent that forms a complex with DNA and then transports it into the cells. Either 200 ng of the pCMV4-AR constructs (16, 22 or 28CAG with 23GGN) or pCMV4 lacking the AR, together with 600 ng of the pGL3-basic vector containing the firefly-luciferase reporter gene were used. The pGL3-basic vector is driven by the human PSA promoter harbouring an enhancer sequence with three AREs. Two of them are located in the proximal promoter whereas the third is found 4 kb upstream of the transcription initiation site. In study I, one of either of the transfection efficiency controls were used; 1 ng of pCH110β-gal or 5 ng pRL-SV40 Renilla. These vectors were used to investigate if the results could be influenced by any of the two common control vectors.

After 24 hours, the media was changed to phenol-red free RPMI or DMEM containing 10% dextran charcoal-treated stripped serum and the same supplements as mentioned above and 10 or 100 nM DHT or R1881 in study I, 10 nM DHT in the presence or absence of CB-153 or 4,4'-DDE in low (1 nM or 1.7 nM) or high 10 nM or 17 nM) concentration, or in a mixture (1x, 10x or 100x high) in study II, 10 nM DHT in the presence or absence of increasing concentrations (1.2 pM, 12 pM or 12 nM) of TCDD in study III and 10 nM DHT in study IV. Media without hormone was used as a control in all studies as well as methanol (II) or DMSO (dimethyl sulfoxide) (III) respectively, because CB-153 and 4,4'-DDE were diluted in methanol and TCDD in DMSO. Methyltrienolone (R1881) was used because it is a synthetic testosterone that is not reduced to DHT. In each experiment, cells were transfected in parallel with pCMV4 without the AR (mock) as control.

The concentrations of CB-153 or 4,4'-DDE used in study II, corresponded to what has been measured in serum lipids from men participating in a study were the aim was to try to confirm the environmental hormone hypothesis and to increase the limited knowledge on human health impact of dietary POPs. In study III, the lowest TCDD concentration corresponded to what has been measured in plasma from Swedish fishermen from the east coast who consume almost twice as much fatty fish from the contaminated Baltic Sea as the general population (Svensson *et al.*, 1995). These men are therefore exposed to approximately 1.2 pM TCDD. The middle TCDD concentration of 12 pM was equivalent to what has been measured in residents living closest to the TCDD manufacturing plant that exploded in Seveso (Pesatori *et al.*, 2009) as well as in cases of Vietnam war Veterans (Gupta *et al.*, 2006). The highest concentration of 12 nM TCDD has been used in previous studies in PCa cell lines (Jana *et al.*, 1999, Morrow *et al.*, 2004). CYP1A1, that is

a target gene of AhR, was induced only at highest concentration and therefore only 12 nM TCDD was used in the PNT1A cells. AR activity was adjusted for AR protein amount.

After 24 hours, the cells were harvested in lysis buffer provided by the manufacturer. Luciferase and β-gal activities were assessed by the Dual Light Luciferase assay system, whereas the combination of luciferase and Renilla were measured with the Dual Luciferase Reporter assay (I). In study II-IV, luciferase was assessed by the Luciferase Assay system. The protein absorbance was determined in duplicates by Quick start Bradford protein assay. The experiments were repeated up to 31 times with DHT and 6 times with R1881 in study I, up to 8 times with CB-153 and 4,4'-DDE at all concentrations in study II, up to 7 times with TCDD in study III and 23 times for COS1 and up to 12 times in the prostate cell lines in study IV.

Quantitative real-time PCR (I, III and IV)

Regular PCR (polymerase chain reaction) is only semi-quantitative and the amount of product is not related to the amount of input DNA. In order to measure messenger RNA (mRNA), this method was extended using reverse transcriptase to convert mRNA into complementary DNA (cDNA) which subsequently was amplified by PCR and analysed by agarose gel electrophoresis. Since there is an extra reverse transcription step, this method is even less quantitative than regular PCR. Therefore, quantitative real-time PCR (qPCR) was developed. In qPCR, quantitation of the amount of cDNA can be determined by measuring at what cycle a certain threshold level is reached (the C_T value) which must be done where the amplification is exponential. The two main methods for determining cDNA amount are based on the use of labelled hydrolysis probes or incorporation of fluorescent dyes that bind non-specifically to the double-stranded DNA.

In study III we measured mRNA expression of CYP1A1 in PNT1A and PC-3 cells, by utilizing a florescent dye (SYBRGreen). SyBRGreen has a high binding affinity to the minor groove of the double-stranded DNA (dsDNA) and when bound to dsDNA the fluorescence increases with every cycle. A generated melting curve, indicates the melting temperature of the products that are amplified, which should be your gene of interest, your endogenous house-keeping control gene that is equally expressed in your sample and sometimes also from non-specific dsDNA such as primer-dimer.

Total RNA was extracted from transfected PNT1A and PC-3 cells, respectively. Preparation of cDNA was carried out by using 1µg total RNA with the oligo $(dT)_{18}$ primer in the RevertAid First Strand cDNA Synthesis Kit. Brilliant II

SYBR Green QPCR Master Mix was used for real-time PCR reactions with 0.3 µM of the following intron/exon spanning primers: CYP1A1 forward 5´-GGC-CGGCGCTATGACCACAA-3´, CYP1A1 reverse 5´-CCGCCGTGACCT-GCCAATCA-3´, β-actin forward 5´-CGTGGGCCGCCCTAGGCACCA-3´ and β-actin reverse 5´-TTGGCCTTAGGGTTCAGGGGG-3´. The PCR reaction was carried out in a MX3000P thermal cycler with an initial denaturation step at 95ºC for 10 min, denaturation at 95ºC for 30 sec, annealing at 57ºC for 1 min and finally an extension step at 72ºC for 1 min. The CYP1A1 mRNA expression was determined by using the comparative C_T method, in which the CY1A1 values where normalised to β-actin as the endogenous control and the median long AR containing 22CAG stimulated with the endogenous hormone DHT, set as the calibrator. Since mRNA of the commonly used endogenous control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been shown to be upregulated by TCDD (Reyes-Hernandez *et al.*, 2009), β-actin was used for normalisation. The experiments were done in triplicates and measured 3 times for each cell line.

The same procedure was performed to measure AR mRNA expression in COS-1 cells in study I and in PNT1A and PC-3 cells in study IV. Primers used for AR detection were, forward 5´AGCCTATTG-CGAGAGAGCTG - 3´ and reverse 5´-GCTTCACTGGGTGTGGAAAT-3´. The endogenous house-keeping gene, GAPDH, was determined with following primer, forward 5´-CGACCACTTTGT-CAAGCTCA-3´and reverse 5´-AGGGGTCTACATGGCAACTG-3´.

Western blot (III)

Antibody-based techniques are commonly used for detection and measuring of target proteins. Those include western blot, solid-phase radioimmunoassay and immunoprecipitation. Western blotting identifies proteins that have been separated from each other according to their size by gel electrophoresis with specific antibodies. The blot is a membrane, almost always of nitrocellulose or PVDF (polyvinylidene fluoride). The gel is then placed next to the membrane and application of an electrical current makes the proteins in the gel to move to the membrane where they adhere. The membrane which then is an imitation of the protein pattern of the gel is consequently stained with primary and secondary antibodies and thereafter developed on an x-ray film or photographed with a digital camera.

In study III, we detected the presence of AhR in PC-3 and PNT1A cells, to be able to investigate the effect of TCDD that is mediated through the AhR. We also identified the presence of AR in the PC-3 cells. The cells where lysed in NP40 buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% NP-40) complemented with

1% of serine protease inhibitor PMSF (phenylmethanesulphonylfluoride) together with 1% protease cocktail inhibitor, run on a 4-10% SDS polyacrylamide gel and transferred to a PVDF membrane. Nonspecific binding of protein was blocked by 3% BSA in Tris-buffered saline containing 0.05% Triton X (TBST) and incubated over night with goat anti-AhR (M-20) polyclonal antibody at 1:150 in 3% BSA/TBST or with a monoclonal antibody against the N-terminal part of the human AR at a concentration of 1:1000 in 5% non-fat milk/TBST. Rabbit anti-goat HRP conjugated secondary antibody was applied in 1:1000 in 0.3% BSA/TBST or peroxidase-conjugated anti-mouse (1:5000) antibody for AR. The proteins where detected using an ECL chemiluminescence kit. Ln-Cap cells were used as a negative control and mouse testis as a positive control. PNT1A and PC-3 cell lysates where loaded in duplicates. β-actin was used as a loading control to assure equal amounts of protein.

Enzyme linked immunosorbent sandwich assay (I, III and IV)

Since western blot is a valuable technique for detecting specific proteins, but a crude method for determining specific protein amount, we used an AR enzyme-linked immunosorbent sandwich assay (ELISA) to determine accurate protein amounts. This method uses two antibodies that each recognises a distinct epitope on the protein of interest. The ELISA plate is precoated with the first antibody, which is used to capture the protein from the sample. Thereafter a second antibody is used to detect the protein bound to the capturing antibody. An HRP (horseradish peroxidase)-conjugated secondary antibody is then attached to quantitate the amount of bound detecting antibody. When the substrate is added, a quantified colorimetric readout is provided.

The ELISA was utilised to rule out that differences in measured AR activity between the three CAG vectors (I, III and IV), depend on varying AR protein amount expressed. The cell lysates were pooled and loaded to a nuclear receptor sandwich AR assay plate and measured three times in duplicates for each cell line. The LnCap nuclear extract in the assay kit and cells transfected with the mock were used as positive and negative controls, respectively. Since there is no purified AR protein available on the market, and therefore no standard curve to determine the exact amount of antigen in the sample, we could only express the relative amounts.

Time-resolved fluorescence imaging (IV)

Detection of serum PSA is regularly used to screen for prostate disease and in follow up of patients with PCa. A high PSA might be an indication of abnormal prostate growth but this does not necessarily indicate PCa. PSA is a downstream

target of AR and its expression is therefore AR regulated. To elucidate whether AR expression and activity varied depending on CAG repeat length, we measured AR and PSA protein amount in human prostate tissue. For this purpose, common immunohistochemistry can be used, but the disadvantages are that the signal obtained is not always linear to protein amount and it often comes with a high background. Instead, time-resolved fluorescence imaging (TRFI) that utilises specific antibodies directly or indirectly linked to lanthanides in combination with image acquisition in an epiflourescence microscope was used. The lanthanides can be excited can be excited at specific wavelengths and have a long decay time. Therefore, it is possible to obtain images with low background noise and autoflourescence. Compared to immunohistochemistry this method is considered to more sensitive and has many advantages such as it gives a linear relation between signal intensity and the specific protein expression and also allows an automated and improved quantification and evaluation of cellular parameters.

Firstly, this method was verified on AR transfected, paraffin embedded COS-1 cells transfected with the three CAG variants and stimulated with 10 nM DHT. The cells were then harvested, fixed and paraffin embedded. Sections were prepared for TRFI and AR protein measurement.

Thereafter, paraffin embedded prostate needle biopsies from Caucasian men were prepared. One sample for each patient was analysed once. The primary antibodies were directed against AR and PSA. They were conjugated to Europium (Eu) and Terbium (Tb), respectively, making it possible to determine both AR and PSA amounts in the same sample. The prostate glands were the primary area of interest, where AR is found in the nucleus of epithelial cells and PSA expressed in the stromal cells could be detected. Since the Eu and Tb emission filters were set at different wavelengths, separate images were obtained with specific signals from exactly the same area. The result was specific density values for each region in every image. Since the mean intensity of the background was subtracted from the region of interest, the images could be compared to each other.

Statistical analyses

Statistical calculations were done by using SPSS statistical package for Windows version 15.0-18.0 and IBM SPSS Statistics 20 as well as the open-source statistical software R version 2.15.0 with the “mgcv” package (R Development Core Team, 2012). All statistical tests were two-tailed with statistical significance defined as $p<0.05$. The selection of statistical test performed was based on the sample size and type of data distribution.

In the *in vitro* studies (I-IV), it was elucidated whether the differences in AR activity between the CAG genotypes depend on the experimental setting, were cell-line specific and whether they were associated with serum PSA. Because of the small sample size, non-parametric Friedman's and Wilcoxon's tests for related samples were utilised. The activity of the most common genotype 22CAG stimulated with 10 nM of ligand (DHT or R1881) was set, as a reference, to 100% and the activities of other genotypes were expressed relative to this. In the case of AR mRNA and protein measured by ELISA, 22CAG was set as 1 and the other genotypes were compared to it. The Friedman test was used for over all comparison of AR function between the different CAG lengths investigated (16, 22 and 28) and the Wilcoxon signed ranks test was used for pair wise comparisons between the reference and the other genotypes.

In the *in vitro* studies with POP exposure (II-III), we also investigated whether the effect of CB-153, 4,4´-DDE and TCDD exposure on AR activity was altered according to CAG repeat length (16, 22 and 28). Primarily, for each set of experiments with all three CAG repeat variants and in each cell line, we evaluated whether POP exposure induced a change in AR activity. By using Wilcoxon's test, AR activity in the absence or presence of POP was compared. Thereafter, AR activity ratios were calculated (TCDD/no TCDD) and compared with Friedman's test. In case of a statistically significant difference, pair-wise comparisons of the ratios were performed (16 vs. 22; 16 vs. 28 and 22 vs. 28) using Wilcoxon's test. The same tests were used to calculate if TCDD could induce CYP1A1 mRNA expression and if this was modified by the CAG genotype.

For the tissue samples in study IV, calculating the diversities between the groups regarding AR or PSA amount, the non-parametric Kruskal Wallis test for independent samples was used for an overall comparison of the three groups of <22CAG, 22CAG and >22CAG. This was followed by a group-wise comparison utilizing the Mann-Whitney test.

To investigate the possible association between CAG number and serum PSA levels in younger and older men in study IV, a univariate regression test was used comparing <22CAG, 22CAG and >22CAG, with 22CAG set as reference. The association with PSA was also tested with the CAG number as continuous variable in partial correlation analyses. Testosterone level was included in the analyses as a confounder.

In study V, we hypothesised that the statistical model could play a role for the association between CAG number and male reproductive function. Therefore, utilisation of an unprejudiced non-linear penalised regression spline analysis with the generalised additive model and continuous CAG as the smooth term was used.

Potential confounders included were abstinence time for semen parameters and BMI for reproductive hormone analyses. However, we also wanted to validate the results in a categorised model with CAG stratified in groups of almost equal size (≤20, 21-23 and ≥24). In case of a linear pattern, CAG number was treated as a continuous variable in a linear regression model. Two cohorts of young Swedish males were included. Primarily, the association between CAG number and semen parameters, testicular volume and reproductive hormones were calculated for each cohort. In order to test whether the data from the two cohorts could be pooled, a statistical test for heterogeneity including significance test for interaction terms between cohort and CAG number in relation to the outcomes was performed in a univariate regression model. No heterogeneities were found and therefore the two cohorts were merged. Semen parameters were assessed in the same laboratory and there were no differences in semen quality between the cohorts (Axelsson *et al.*, 2011). Since the reproductive hormones were measured in two different laboratories, cohort was considered as a potential confounder.

In study V, we also wanted to investigate whether the CAG number could modify the effect of smoking on semen parameters, testicular volume and reproductive hormone levels. For this evaluation only the pooled data from both cohorts was used. The subjects were divided into non-smokers and smokers. The generalised additive model as well as univariate regression analysis with stratified CAG number was used to investigate the association with CAG number and the outcomes for non-smokers and smokers, respectively. Subsequently, interaction terms between CAG and smoking were included in the univariate model; with CAG number both stratified and as a continuous variable, to further investigate the potential of the genotype as a modifier of the effect of smoking on the reproductive parameters.

RESULTS AND DISCUSSION

An inverse relationship between CAG repeat number and AR function was anticipated at the start of this thesis. This was based on a few previous *in vitro* findings (Beilin *et al.*, 2000, Chamberlain *et al.*, 1994, Tut *et al.*, 1997) as well as clinical findings in men with Kennedy's syndrome, having an abnormal expansion to >40 CAG (La Spada *et al.*, 1991). The longer the expansion, the worse was the clinical outcome in these patients (La Spada *et al.*, 1992). Most associations between CAG number and reproductive parameters and pathological conditions have therefore been analysed in a linear model.

Role of the CAG length on AR function and PSA expression (I and IV)

In study I, we investigated whether the assumed negative linear relationship was dependent of the experimental setting. This was done by constructing and utilising AR vectors containing CAG numbers within the normal range (16, 22 or 28) in a cell-reporter assay. The length of the GGN number was held constant at 23 repeats. Due to the fact that the topic was controversial, the approach was to perform sufficient number of experiments to avoid that our results could be to be questioned due to few experiments or regarded as by chance findings. We also wanted to have a more biologically approach by using a physiological PSA promoter in the reporter vector, instead of the rat probasin or promiscuous MMTV (mouse mammary tumour virus) promoter, where the later has shown to be activated by GR and PR receptors as well (Simon and Mueller, 2006).

We found that when correcting for β-galactosidase as a transfection efficiency control, the vector with 22CAG had the highest activity (set as 100%) compared to 16CAG (mean 78%, p=0.0005) and 28CAG (68%, p=0.006). However, the negative control vector, absent of the AR gene, showed considerable high β-gal background activity (86%). When the second frequently used control vector Renilla was used, no differences in AR activity were found between 16CAG and 22CAG, whereas the 28CAG variant had lower activity (104%, p=0.7 and 59%, p=0.004, respectively). In this case, the empty vector displayed 17% compared to the reference.

The high background activity presented for β-galactosidase could not be explained by expression of AR mRNA or protein in the empty vector, as tested in qPCR and ELISA. Previous reports have shown that the activity for both β-galactosidase and Renilla can be suppressed or enhanced by co-transfected plasmids (Farr and Roman, 1992 , Mulholland *et al.*, 2004). Renilla activity has also been suggested

to be dependent on the promoter driving its expression and on the androgen responsiveness of the cell lines used (Mulholland *et al.*, 2004). Since the luciferase reporter gene expression is dependent on functional AR protein and not only on the transfection efficiency, we adjusted for AR protein measured by ELISA.

Adjusting luciferase amount for AR protein gave the highest activity for the 22CAG variant compared to 16CAG and 22CAG (20% and 12% thereof, $p<0.0001$ for both comparisons). The same pattern was seen when corrected for total protein, where 16CAG and 28CAG obtained 29% and 18% ($p<0.0001$ for both) of the activity compared to 22CAG. A similar pattern was found when stimulating the cells with 100 nM DHT, indicating that the effect was not dependent on the hormone concentration. The same experiments done with 10nM and 100nM R1881, demonstrated the same pattern as with DHT, specifying that the CAG dependent effect on AR activity is not dependent on the type of androgen present. This indicates that adjusting for total protein amount, giving an AR activity pattern close to that of adjusting for ELISA measured AR protein in the COS-1 cells, might be a more cost-efficient way of handling the data.

Adjusting for AR protein amount was also suggested by a former study in PCa DU145 cells, lacking AR expression, where CAG lengths from 14 to 25 were transfected together with a reporter vector harbouring a human PSA promoter to study the effect on AR function (Ding *et al.*, 2004). However, AR protein amount was measured with western blot that is a crude method, and might not be specific enough to measure the small differences in AR protein between the CAG variants expected. A statistically significant overall difference in AR protein was found, but due to few replications of the experiments, no specific differences between the CAG repeat variants were detected. A statistically significant difference in AR protein adjusted activity between 15CAG and 17CAG compared to 21CAG was found, which was used as reference. However, there was no consistent pattern in AR activity and all vectors harboured 16GGC (22GGN), repeat lengths that is shorter than the mean number sequenced in populations (Kittles *et al.*, 2001).

In speculating about why both short and long CAG alleles not would be of disadvantage, it has to be take into consideration that variation of the CAG encoding glutamine tract is determined both by definite sequence features and its specific role within the protein's structure and function (Butland *et al.*, 2007). This indicates that it is critical to maintain a particular CAG repeat length in these proteins. In the case of the AR, a range of approximately 10-30 repeats seems to be tolerated for proper function and has also been shown to be balanced by both AR mRNA and protein as shown in our first study. Repeat length ranging from 16 to 29 repeats have *in vitro* been shown to exhibit high transactivation capacity and

strong N/C interaction, necessary for protein activation and interaction with coregulators (Buchanan *et al.*, 2004). Since between 91-99% of the AR gene CAG repeat alleles in different ethnic groups have between 16-29CAG, the authors suggest that this might be the critical range preserved as an essential component of androgen-induced AR signalling. It might also be that alleles outside this CAG repeat range would be a more important mediator for disease phenotype than a linear increase in CAG length. This approach has contributed to further interest in the field and has been debated in recently published review article (Krausz, 2012).

Thus, the relation between CAG number and AR activity may not be linear as previously expected. An alternative way to analyse the data would therefore be through stratification, or to use an unprejudiced non-linear regression model to investigate the impact of CAG number on disease predisposition. Stratifying the CAG number has in a recently published study on risk of subfertility with nearly 4000 subjects resulted in an association between short and long CAG numbers and increased odds of subfertility was found (Nenonen *et al.*, 2011).

Since PSA in synthesised and secreted by epithelial cells of the human prostate in response to androgens, its expression can be used as a measure of AR activity. In study IV, the aim was to examine whether PSA concentration in serum of young Swedish men (n=187) and elderly Norwegian men (n=172) who had no priori diagnosis of prostate disease, as well as amount in non-malignant prostate tissue, are CAG number dependent. Because of the previously demonstrated non-linear pattern of CAG number on AR activity, the men were stratified into groups with <22, 22 or >22CAG. We found that men with the median number of 22CAG repeats had the highest PSA both in the young (17-21) years and elderly men (60-80 years). Younger men carrying 22CAG displayed a 26% higher mean PSA level than men with <22CAG (mean 0.86 ng/ml vs. 0.64 ng/ml, p=0.04). Compared to men with >22CAG (0.59 ng/ml), the reference group had 32% higher PSA (p=0.02). In the older men, carriers of 22CAG had 60% higher mean PSA in their circulation, compared to men with shorter repeats (5.35 ng/ml vs. 3.24 ng/ml, p=0.08). In comparison to men with longer repeats (2.22 ng/ml), those in the reference group had 59% higher PSA serum level (p<0.003). All results were unchanged after adjustment for testosterone concentrations. In summary, carriers of the average CAG repeat length of 22 had the highest PSA concentration in their circulation compared to shorter and longer repeats. However, the low number of men in the 22CAG group is a limiting factor and the reason for lack of statistical significance for all comparisons and these findings should be replicated in a larger cohort.

The same pattern in AR transactivation as for COS-1 cells was found in PNT1A and PC-3 cells, with 22CAG having the highest activity compared to the 16CAG and 28CAG variants ($p<0.05$ for all comparisons). The results are displayed in Figure 6.

The PSA amount in benign areas of prostate tissue samples was also highest in those with 22CAG. Regarding the mRNA and protein levels, the 22CAG variant expressed the lowest amount in both prostate cell lines, in relation to the shorter and longer variant ($p<0.05$ for all comparisons). This indicated that even in an experimental setting, differences in function can be compensated for by higher AR expression. The same pattern was recurring in the tissue samples. However, probably due to the small sample size (n=19) and high sample-to-sample variation, none of the differences between the prostate tissue samples were statistically significant ($p>0.8$). The reason for this was that few men in the study group were carriers of very short and very long CAG repeats, respectively. Despite the widespread clinical use of PSA as a tumour marker, only a few studies have examined the relationship between serum PSA level and AR CAG number. A negative association between serum PSA and CAG repeat length as well as a positive association between CAG number and testosterone has been reported in subfertile men with defective spermatogenesis (Mifsud *et al.*, 2001a). This was not found in fertile men indicating that the androgenic environment plays a role for these associations. Likewise, an inverse association between PSA found in seminal plasma and CAG number has been found in young men. Another study that was

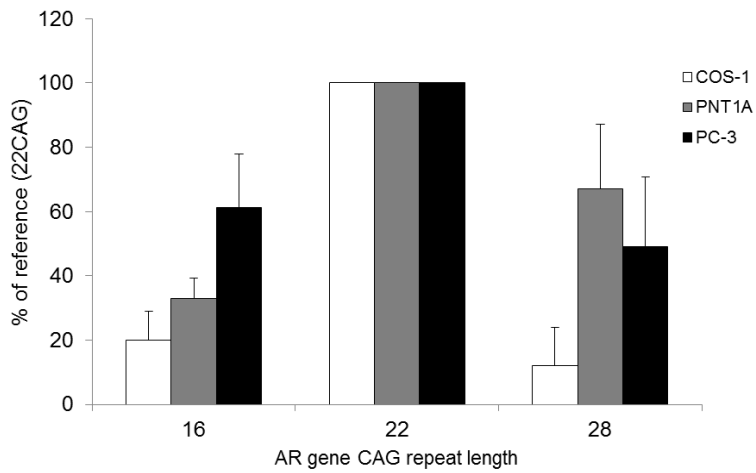

Figure 6. *Androgen receptor activity in COS-1, PNT1A and PC-3 cells, adjusted for AR protein amount. The cells were stimulated with 10 nM DHT and 22CAG was set at as a reference at 100% for each cell line.*

not based on the assumption of a linear relationship between CAG number and AR activity as the others, dividing CAG repeat length into quartile groups (≤19, 20-21, 22-23 or >23), did not find an association with serum PSA. However, these men were already diagnosed with advanced PCa that might contribute to loss of normal androgen regulation and together with the other studies had no information about ethnicity making generalisation difficult, while the CAG repeat distribution is known to exhibit ethnical differences.

To our knowledge, the 22CAG variant has not been investigated separately and linked to increased risk of prostate disease. Men carrying this genotype, that naturally have higher PSA than counterparts with shorter or longer repeats, may have an increased risk for PCa investigation and are subsequently at higher risk to be selected for prostate biopsy. On the other hand, those with shorter and longer CAG number could be at risk for being undetected in a PSA screening test until incurable state. Powell and colleges have shown that men with >18CAG repeat length had a greater risk of PCa reoccurrence compared to men with ≤18CAG, demonstrating that disease progression after prostatectomy was associated with CAG number (Powell *et al.*, 2005).

From two large studies on mortality results from randomised PCa screening trials the conclusion was that, even though one of the studies PSA screening reduced the death of PCa with 20%, it was associated with high risk of overdiagnosis that is defines as the diagnosis in men who would not have clinical symptoms (Andriole *et al.*, 2009, Schroder *et al.*, 2009). This warrants an area of further investigation on whether the CAG genotype could be included in the individualised diagnostic procedures when setting the cutoff levels in PSA screening. The outcome of studying this association in a larger population might result in reduction of the number of histologically negative biopsies and thereby preventing unnecessary invasive procedure and in turn improve the quality of life.

Modifying effects of CAG number of POPs on AR activity (II and III)

Since exposure to EDCs, such as POPs, has been suggested as strong candidates for interfering with the male reproductive system and that some populations seem to be more susceptible to the adverse effect than others, we wanted to investigate if the impact of some of these compounds on AR activity was modified by the CAG genotypes. This association has been shown in an epidemiological study on men differently exposed to POPs, where those with <20CAG repeats in the AR and highly exposed to PCBs as measured with the PCB marker, CB-153, had a 35% reduction in sperm concentration (Giwercman *et al.*, 2007). Experimental studies have shown that 4,4'-DDE can inhibit androgen binding and androgen induced

transcriptional receptor activity (Kelce *et al.*, 1995), whereas the impact of CB-153 on AR transcriptional capacity is not clear since some have found an association (Schrader and Cooke, 2003) and others not (Bonefeld-Jorgensen *et al.*, 2001).

In study II, we investigated any possible modifying effects of CAG number of exposure to the PCB congener and the 4,4'-DDT metabolite, CB-153 and 4,4'-DDE, on DHT-induced AR activity in COS-1 cells, as single compounds and in a mixture. No CAG number dependent relative changes in response to low concentration of CB-153 or 4,4'-DDE were found. When comparing the activity after CB-153 exposure to that with DHT only between the CAG variants, pair-wise comparisons showed that the effect of high CB-153 on the 28CAG repeat variant differed from that on 16CAG and 22CAG ($p=0.03$ and $p=0.04$, respectively). However, when investigating the relative CB-153 induced activity for each CAG length separately, these differences were not statistically significant. When studying the 4,4'-DDE induced relative change in AR activity between the three lengths after high exposure, the 16CAG repeat variant had a lower relative activity as compared to 22CAG and 28CAG ($p=0.02$ and $p=0.06$, respectively). Comparing the impact of 4,4'-DDE on each CAG length separately, the activity of the 16CAG variant was also statistically significantly hampered ($p=0.02$) compared to cells treated with DHT only, whereas the other two variants were unaffected. No effect on AR activity was noted without DHT present, indicating the absence of agonistic properties of the POPs. The POP induced relative AR activity changes are presented in Figure 7.

Since the *in vivo* exposure includes a combination of several compounds, we also investigated the effect of mixtures of the two compounds on AR activity. The 28CAG variant was hampered in activity after exposure to 1x the mixture, both compared to the other variants (16.vs. 28 $p=0.02$ and 22 vs. 28, $p=0.03$) and compared to the activity of DHT only on each length separately ($p=0.02$). Higher doses of the mixture were also tested. Ten times the mixture did not induce any relative changes in AR activity compared to cells treated with DHT only, whereas 100 x the mixture hampered the activity for all CAG variants. This was not due to cell death, since the cells were visually well-doing before harvest and no differences in total protein were detected.

Since the *in vitro* system also has its drawbacks, the results from this study cannot be directly compared to the *in vivo* situation where a lot of other physiological factors as e. g. the influence of other sex steroids, hormone metabolism and the HPG-axis may have an impact on the androgenic activity. On the other hand, in epidemiological studies there are many other confounding factors that can affect

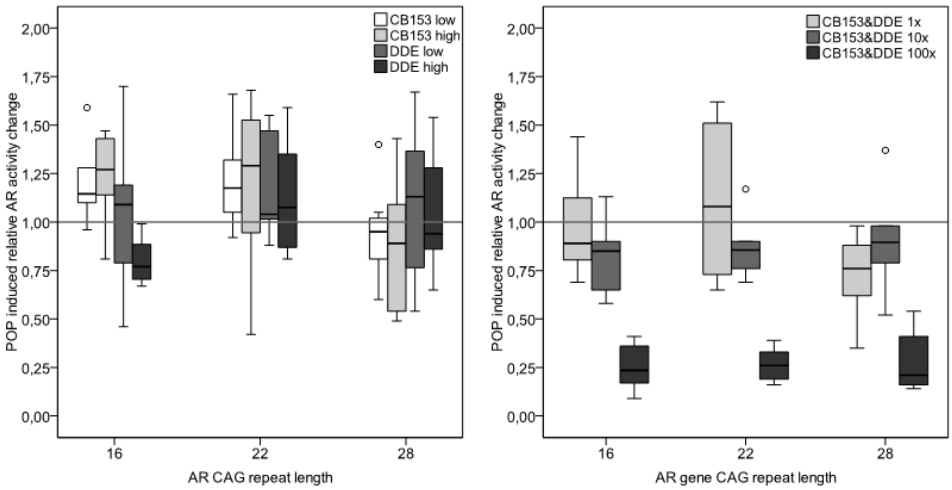

Figure 7. *The relative change in DHT-induced AR activity in COS-1 cells exposed to CB-153 or 4,4'-DDE in low or high concentration, or in a combination at 1x, 10x or 100x the high concentration. Boxes represent values to the inter-quartile range (25^{th}-75^{th} percentile) with a median line and whiskers to the minimum and maximum values. Outliers that are 1.5-3 times the interquartile range are marked with ° and extreme values (>3 times the interquartile range) with *. The baseline, set at 1, defines the AR activity in response to DHT only.*

the outcome for an association found such as other genetic factors, environmental exposures and life-style habits. One discrepancy between these studies is that our experimental data show that the AR variant containing the shortest CAG repeat indeed displayed hampered relative activity but in contrast to the epidemiologic result, this was in response to high 4,4'-DDE exposure and not to CB-153. An explanation for these results could be the high correlation between CB-153 and 4,4'-DDE (Rignell-Hydbom *et al.*, 2004) or that other congeners that are associated with both compounds exerted the effect in the epidemiological study. Nevertheless, this study together with the epidemiological finding contribute to strengthening the hypothesis that the AR CAG repeat length might modify the effect of POPs on male reproductive function. It should also be mentioned that in the experimental study, the modifying effects were only seen at exposure levels comparable to those found in humans, whereas these effects were absent at the higher concentrations tested.

In this *in vitro* study, a heterogeneous effect of the compounds, where the short CAG variant was affected after single exposure to 4,4'-DDE, whereas the long variant was hampered in activity after exposure to both POPs was revealed. A dose-dependent negative response of the mixture was also found. These effects

could either be due to intervention with the transcription machinery, or a result of an inhibitory effect at the RNA-level and in turn preventing protein translation. Coregulators interacting with the N-terminal CAG repeat might be important players in those processes. The compounds could possibly also promote degradation of the AR protein in a dose dependent fashion. POPs have previously been reported to induce AR proteasomal degradation through interaction with the AhR (Ohtake *et al.*, 2007). However, since the COS-1 cells do not express the AhR (Kollara and Brown, 2006) this cannot be the case, which however does not exclude AR protein degradation through some other mechanism.

Even though some POP combinations hampered AR activity in a CAG length dependent manner, the overall activity pattern with the 22CAG variant having the highest activity compared to the other lengths was not changed after exposure (Figure 8). This indicates that the most common allele may be more resistant to adverse effects of these kinds of compounds, compared to the less common variants.

In study III, we wanted to elucidate whether TCDD had a CAG length dependent effect on AR function in PNT1A and PC-3 cells. This was based on the findings that men exposed to TCDD-contaminated Agent Orange have been reported to shown to have a higher incidence and a more aggressive variant of PCa, compared to unexposed men (Chamie *et al.*, 2008). The finding that these men also are likely

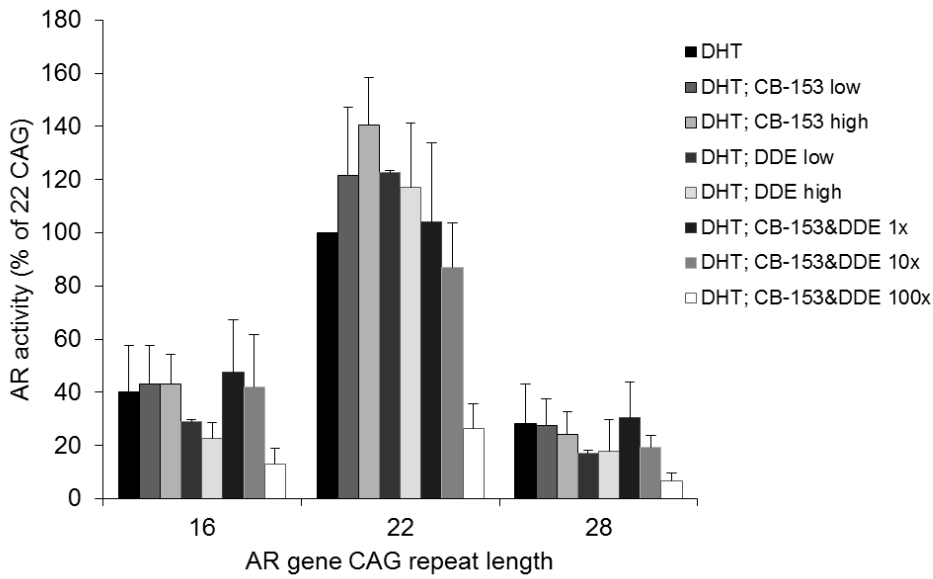

Figure 8. *The DHT-induced AR activity at all POP concentrations in COS-1 cells, separately or in combination.*

present with lower preoperative PSA concentrations in the circulation before radical prostatectomy (Shah *et al.*, 2009), indicates an interaction between AR regulation and the AhR that mediates the effect of TCDD. Due to that African Americans have the highest incidence and mortality rate from PCa, together with in average shorter CAG repeat length in the AR (Bennett *et al.*, 2002, Lange *et al.*, 2008), the possible association between CAG repeat length and PCa have been extensively studied.

No TCDD induced changes in DHT-induced AR activity were found in the PC-3 cells. However, in PNT1A cells, the 16CAG variant had a higher relative activity after exposure to 12 nM TCDD related to 22CAG and 28CAG ($p=0.046$ for both). Comparing the impact of TCDD on each CAG length separately, the activity of the 16CAG variant was also statistically significantly induced ($p=0.03$) related to cells treated with DHT only, whereas in that manner the other two variants were unaffected. The TCDD induced AR activity change is shown in Figure 9.

In agreement to previous results in COS-1 cells, the 22CAG variant expressed the least AR protein as compared to the shorter and longer variants when stimulated with 10 nM DHT in both cell lines and the same pattern was seen after TCDD exposure ($p<0.05$ for all).

The western blot demonstrated the presence of AhR in both cell lines, a prerequisite for discussing a possible AR-AhR cross-talk. However, no differences in CYP1A1 mRNA expression were found, excluding an effect of the CAG repeat length on AhR activity at that level.

The increased activity for the 16CAG variant in the non-malignant PNT1A cells corresponds to what has been found in a previous study in COS-1 cells (Kollara and Brown, 2010). The same study also included PC-3 cells, where no TCDD induced effect on neither proliferation nor DHT-induced AR activity was found. This could be due to loss of normal androgen regulation in the PCa cells and a lost sensitivity to TCDD, although our explorative study cannot confirm such a hypothesis. There have been conflicting opinions on the role of AhR in tumourogenesis, where some have suggested suppressor properties (Fritz *et al.*, 2007) whereas others claim that since AhR regulates phase I enzymes that convert carcinogens to active genotoxins it act as a tumour initiator (Dietrich and Kaina, 2010). This has led to speculations regarding dose, and that low AhR signalling may protect against PCa, while high AhR signalling might promote it (Mehta and Vezina, 2011). Since the dose we used in our experimental study was above those measured in humans, indicates that the results should be taken with some caution.

Since recent reports have demonstrated that TCDD-induced AhR can cause a decrease in AR protein levels (Ohtake *et al.*, 2007), we speculated that the

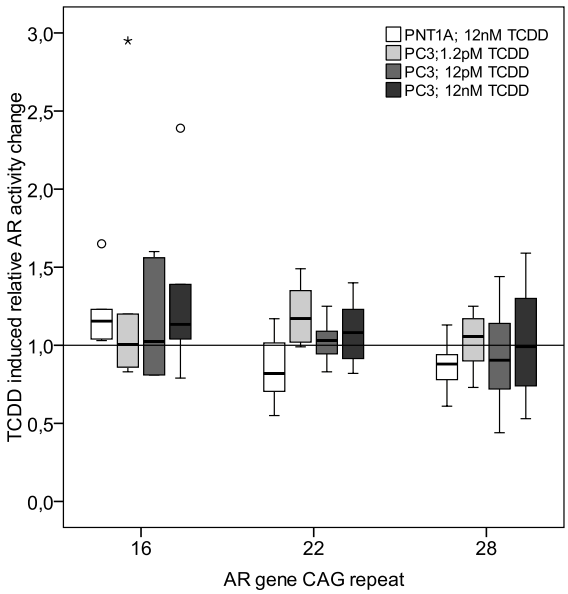

Figure 9. *Relative change in DHT-induced AR activity in PNT1A cells treated with 12 nM TCDD or PC-3 cells treated with 1.2 pM, 12 pM or 12 nM TCDD.*

increased activity for the 16CAG repeat variant induced by TCDD might be due to the constitution of the AR receptor. However, our study could not confirm this hypothesis since addition of TCDD did not change the protein amount for AR containing 16CAG in PNT1A cells. Instead, TCDD contributed to increased AR protein expression for the 22CAG variant. The diverging results may be a consequence of the different cell lines used and the unknown CAG lengths in previous studies. Another possibility for the increased activity of the short CAG repeat variant could be an AhR-independent effect of TCDD. Even though almost all effects of TCDD are mediated through the AhR, there have been some genes reported to respond to TCDD in an AhR-independent manner (Tijet *et al.*, 2006) and the principal pathways of TCDD-induced antiproliferation in breast cancer cells have suggested to be AhR independent (Yoshioka *et al.*, 2012). A study in CHO cells, that do not express AhR, demonstrated hampered AR activity after exposure to selected PAHs (Vinggaard *et al.*, 2000). Even though the anti-androgenic effects by PAHs observed were suggested to be due to a direct binding to the AR or binding by one or more metabolites of the PAH to AR, this further adds evidence that there are critical target molecules other than AhR for the toxic actions of TCDD.

The TCDD-induced increased activity for the 16CAG variant that was found in this study was a relative change from its initial position. The 22CAG variant still

had higher absolute activity, although the difference in activity between the receptor variants became smaller in the presence of TCDD. This result is similar to the previous study and contributes to further support that CAG lengths around the median length may most robust in response to POPs.

Association between the CAG polymorphism and reproductive parameters and it's interaction with smoking (V)

The non-linear relationship between CAG repeat number and AR activity found in our studies might explain why the associations between CAG number and reproductive function have given inconclusive results. Since the statistical model used seems to have a large impact on the outcome of these kinds of studies, utilisation of an unprejudiced non-linear penalised regression spline analysis with the generalised additive model and continuous CAG as the smooth term probably would be a more proper strategy. This was tested in study V, in which the data also were investigated a categorised model with CAG stratified in groups of almost equal size (≤20, 21-23 and ≥24). In case of a linear pattern, CAG number was treated as a continuous variable in a linear regression model to be able to compare our results with previous findings. Two cohorts of young Swedish males were included (Cohort A: n= 224 and Cohort B: n=314) and analysed both separately and combined.

Since none of the associations between CAG number and outcomes could be replicated in both cohorts, they were merged to get a larger study material. In the spline analysis, statistically significant associations between CAG number and testosterone (p=0.04) as well as with LH (p=0.02) were found (Figure 10). When further analysed in the categorised model, we found that men with 21-23CAG repeats also had 6% higher testosterone than those with ≤20CAG repeats (21 nmol/l vs. 20 nmol/l, p=0.02). In the linear model, however, men with longer CAG had increased levels of both testosterone (linear regression coefficient β: 0.142, p=0.03) and E2 (β: 0.707, p=0.009). This is in line with previous findings from a study on nearly 3000 men, 40-80 years of age, from eight different European countries (Huhtaniemi *et al.*, 2009), where it is suggested that the phenotypic correlations between CAG number and osteoporotic fracture risk as found in their study, together with previous associations with outcomes such as HDL (high-density lipoprotein)-cholesterol, body fat and leptin, might actually be due to differences in oestrogen instead of androgen action. This might explain part of the difficulties in finding a link between CAG number and reproductive parameters, as variations in receptor activity may be blurred by parallel changes in the levels of both testosterone and oestrogen in addition to the statistical model used.

The categorised analysis also revealed that men with ≥24CAG had 15% (3.1 IU/l vs. 3.6 IU/l, p=0.009) lower FSH levels compared to men with ≤20CAG. This pattern differs from the smooth curve in the spline model that does not show any association with CAG number in general, except for those few men with a genotype that included more than 30 CAG repeats in the AR gene. Thus, high CAG number, even within the normal range, seems to have an effect on the HPG-axis already in young age.

Furthermore, since smoking is highly prevalent in the general populations and reduced sperm number, sperm concentration, semen volume and motility as well as decreased levels of FSH and increased levels of LH and testosterone has been found in smokers compared to non-smokers (Ramlau-Hansen *et al.*, 2007a, Richthoff *et al.*, 2008), we wanted to investigate whether the CAG repeat length could modify the effect of smoking on semen parameters, testicular volume and reproductive hormone. Compounds that are present in cigarette smoke, such as the PAHs including B[a]P have in experimental studies shown to have anti-androgenic effects by inhibiting AR activity (Kizu *et al.*, 2003, Vinggaard *et al.*, 2000), decrease PSA mRNA and protein level (Kizu *et al.*, 2003) and to promote degradation of the AR (Ohtake *et al.*, 2007).

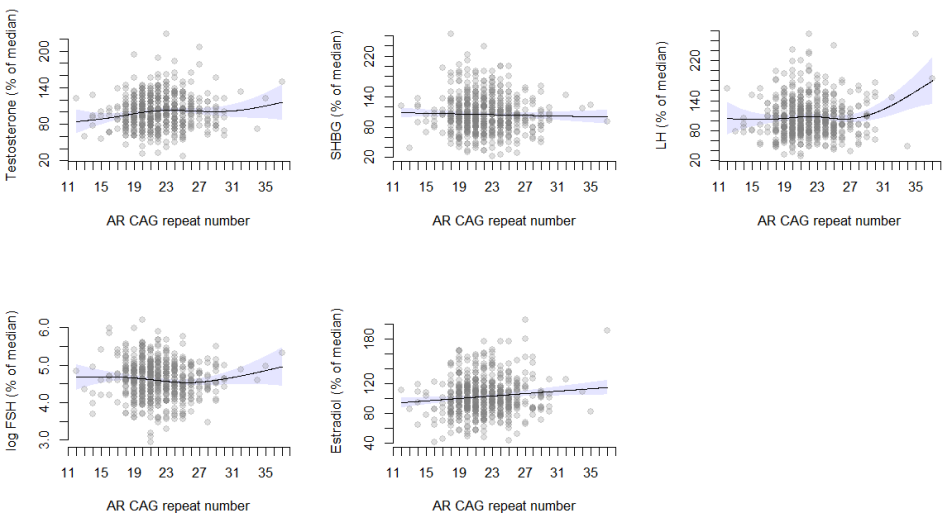

Figure 10. *Associations between CAG number and testosterone, sex hormone-binding globulin (SHBG), luteinizing hormone (LH), log Follicle stimulating hormone (FSH) and oestradiol in non-linear regression splines, adjusted for BMI. The data are expressed as % of median from each cohort. The shaded area in each plot represents the 95% CI and the line represents the smooth curve for all men.*

We found a modifying effect of CAG number on semen volume in smokers compared to non-smokers in the spline analysis as seen in Figure 11. (p=0.02). This was confirmed in the categorised model where smoking men with ≥24CAG had 20% lower semen volume than those with ≤20CAG (2.4 ml vs. 3.0 ml, p=0.03) as well as in the linear regression model (β:-0.08, p=0.02). A borderline statistically significant interaction between CAG number and smoking on semen volume was also found (p=0.08). Since the seminal fluid predominately consists of prostate- and seminal vesicle fluid, it is possible that the CAG length dependent differences in AR function are of larger importance in DHT-dependent organs like the prostate and seminal vesicles in men exposed to cigarette smoke, than in less DHT-dependent tissues. It has been shown that the cross-talk between AR and AhR presenting decreased PSA mRNA and protein level (Kizu *et al.*, 2003) and labeling AR for degradation (Ohtake *et al.*, 2007) is dependent on DHT, that is locally reduced from testosterone to DHT in the prostate.

No statistically significant modifying effects or interactions between CAG number and maternal smoking were found on the reproductive outcomes. This does not exclude a damaging effect of maternal smoking during pregnancy on the reproductive function of their sons, but suggest that in this matter there are other factors than the CAG number that may contribute to the individual susceptibility to the harmful compounds present in cigarette smoke.

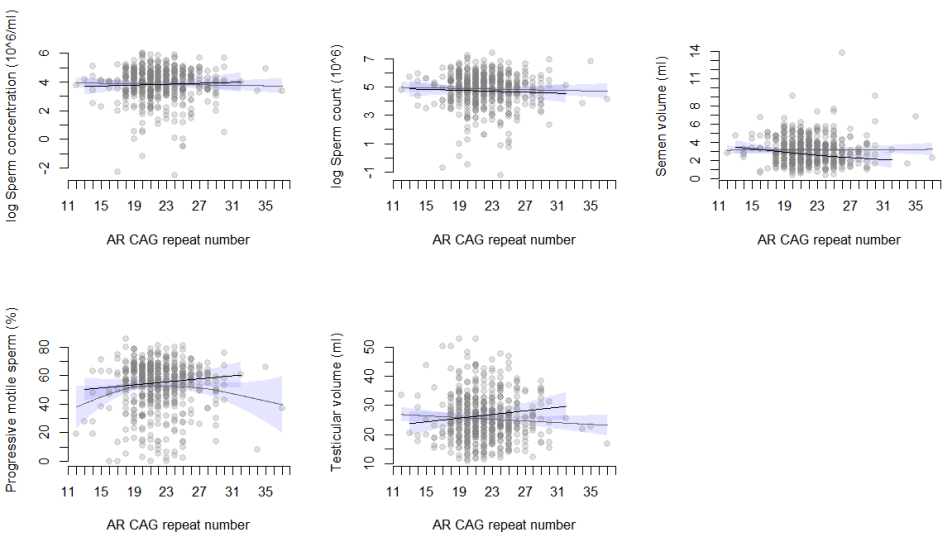

Figure 11. *Associations between CAG number and log sperm concentration, log sperm count, semen volume, progressive motile sperm, testicular volume for smoking (black line) and non-smoking (grey line) men, adjusted for log abstinence time.*

CONCLUSION

These studies contribute to reveal the relationship between AR CAG repeat number and AR function, as shown both in experimental settings and *in vivo* by using a new statistical approach on men from the general population. The results in this thesis also suggest that the fine-tuning effects of CAG polymorphism on AR function and reproductive parameters, in combination with other genetic, environmental and life-style factors, may contribute to the susceptibility to POPs.

Conclusions from the *in vitro* and *in vivo* studies are that:

- The relationship between AR CAG repeat length and AR activity is not linear as previously assumed, but follows the distribution in populations, with the most common variant having the highest activity. AR mRNA and protein amount were lowest for the variant with the highest activity, indicating that even in an experimental setting; differences in function can be compensated for by AR expression.
- Men having the median CAG number in their AR had the highest serum PSA concentration and prostate tissue PSA amount, compared to shorter and longer lengths, indicating a higher AR activity for this genotype.
- Exposure to POPs had dose- and CAG-dependent effects on AR activity in non-malignant cell lines. However, the effects were only seen on the short and long CAG repeat variants and the overall activity pattern with the most common variant having the highest activity compared to the other lengths was not changed after exposure. This indicates that alleles in that range may be more resistant to adverse effects of these kinds of compounds, compared to the less common variants.
- Utilisation of an unprejudiced spline regression analysis gives a better picture of the relationship between CAG number and reproductive parameters and hormones than using only a linear model.
- The AR CAG repeat length was associated with reproductive hormone regulation, most assumedly through the hypothalamo-pituitary-gonadal axis. Smoking men with longer CAG number had a lower semen volume than smokers with shorter CAG variants.

FUTURE PERSPECTIVES

Future studies would focus on the interaction between AR CAG repeat length and coregulators. By using different promoters and performing DNA binding studies, it could be elucidated. It would furthermore be of interest to test other CAG repeat variants in the common rage such as 20, 21 and 23 on AR function. Additionally, this may be done with varying GGN repeat lengths.

The effects of POPs on male reproductive health are difficult to predict, as they are most probably dependent on the mix of substances, timing of exposure, individual genetic susceptibility, and complexity of the interactions of signalling pathways that are activated or inhibited, therefore much more work is to be done. A start would be to further investigate the CAG number as a modifier of the association between POP exposure and AR function *in vitro*, with or without AhR. This could be done by adding other mixtures of compounds in increasing concentrations for dose-response effects and time-dependent effects. DNA binding studies to investigate if the compounds may impact the binding to the AREs in the androgen responsive genes depending on the CAG repeat length can also be performed. We started to investigate whether the interactive effect between AhR and AR is DHT-dependent or if similar effects can be found with R1881, which could be followed up.

Further experiments to be performed is to add siRNA to knockdown the AhR and find out if it abolishes the TCDD induced effect on the short CAG variant in the PNT1A cells, which could not be explained by a change in AR protein level. Proteasome inhibitors to study variations in AR protein expression, with and without TCDD, can also be added. Furthermore, to study this effect in additional cell lines to find out whether the alterations in the regulation of cell processes related to malignant transformation can play a role in the lost sensitivity to TCDD would be interesting. The interaction could also be studied *in vivo*, by introducing established stably transfected cells with the CAG variants into a mouse model and expose them to TCDD, to give a picture of the effects in a whole organism.

The spline regression model could be used for associations of CAG repeat number with PCa and infertility in further studies.

POPULÄRVETENSKAPLIG SAMMANFATTNING

De senaste årtiondena har det diskuteras om det finns anledning till oro när det gäller mannens reproduktionsfunktion. Vissa forskningsresultat tyder på att spermieantalet har minskat och fler män har diagnostiserats med testikelcancer och medfödda missbildningar av könsorganen än tidigare. Om detta beror på genetiska faktorer, livsstil eller miljöfaktorer, eller alla tre är ännu inte känt. Orsksaken till symtomen har föreslagits vara hormonstörande ämnen, även kallade miljögifter. Många av dessa ämnen är fettlösliga och svårnedbrytbara. De ackumuleras i näringskedjan och även om många av ämnena är förbjudna i de flesta länder finns de kvar i miljön. Människor utsätts huvudsakligen genom mat, men också efter industri-utsläpp, cigarettrök och malariabehandling.

Vissa av dessa ämnen kan ”härma” de manliga könshormonerna; testosteron (T) och 5α-dihydrotestosteron (DHT) och därmed rubba hormonbalansen, eller bryta ner hormonernas mottagare androgenreceptorn (AR). Hormonerna är nödvändiga för normal könsutveckling, pubertet och för reglering av spermieutveckling hos män. De binder till AR, som förmedlar den hormonella effekten via reglering av andra gener. Fel i receptorn resulterar i dålig ”signalöverföring” och kan orsaka sjukdomar med allt från allvarliga missbildningar, som medför att det kan vara svårt att bestämma barnets kön, till lättare varianter som manlig infertilitet.

AR-genen ligger på X-kromosomen, vilket innebär att män bara har en kopia medan kvinnor har två. Detta leder till att genmutationer eller förändringar av AR-proteinets funktion oftast drabbar män. I AR-proteinet finns en sträcka med repeterande DNA-baser som varierar mellan individer och populationer. Dessa baser, CAG, kodar för aminosyran glutamin, och antalet repetitioner har betydelse för proteinets funktion.

En studie på män från fyra länder som var olika utsatta för miljögifter, visade att de som var mest exponerade för PCB (polyklorinerade bifenyler) och hade en viss genotyp dvs. genetisk uppsättning av CAG-repetitioner, hade färre spermier än de som var mindre exponerade. Då kan man fråga sig om dessa män har en ökad känslighet för miljögifter och om de kanske kan förändra funktionen av AR. Detta undersöktes i ett cellsystem med njurceller från apa, vilka inte har någon egen produktion av AR. Vi kunde därför föra in tre olika genotyper för att undersöka vad som hände med AR-proteinets aktivitet när vi tillsatte PCB och DDT, separat eller tillsammans, i olika koncentrationer i närvaro av könshormonet DHT. Vi såg att motsvarande genotyp som i studien på män, var den som påverkades mest av DDT. Mixen hade störst påverkan på en av de andra genotyperna. När man tillsatte en extremt hög koncentration av mixen försvann skillnaderna mellan genotyperna

och AR-aktiviteten minskade drastiskt. Vi såg även att den genotyp som är vanligast bland vita män (22CAG) hade högst aktivitet i närvaro av DHT, både med och utan miljögifterna. De vanligast förekommande genotyperna har visat sig att ha bäst proteinfunktion och har därför föreslagits vara bevarade genom evolutionen, kanske genom att också vara mest motståndskraftiga mot främmande ämnen. Mönstret var likadant när vi testade genotyperna i prostatacellinjer, med och utan att vara angripna av cancer, först enbart med DHT och sedan med dioxinvarianten, 2,3,7,8 tetrachlorodi-benzo-p-dioxin (TCDD).

Män som har varit högutsatta för TCDD har visat sig ha en ökad risk för prostatacancer (PCa). Dessa män har även färre CAG-repetitioner i AR, vilket också har relaterats till risken för PCa. Vi fann att genotypen med färre CAG-repetitioner påverkades mest av TCDD i celltypen utan cancer, vilket gör fyndet intressant. Effekten av TCDD förmedlas genom arylhydrocarbonreceptorn (AhR), även kallad dioxinreceptorn, som kan interagera med AR. Man måste dock ha i åtanke att det är ett cellsystem och i en människa kan kroppen kompensera för en sämre proteinfunktion med att slå på ökad hormonproduktion och jämna ut effekten så länge kroppen fungerar normalt.

Förutom effekten av miljögifter har vi studerat om en mål-gen för AR-proteinet, prostataspecifik antigen (PSA), som används för att diagnosticera PCa, ändras i blodet beroende på genotyp. Vi såg att män med 22CAG-repetitioner hade högst PSA jämfört med män med andra varianter. Detta stämde även överens med mängden PSA-protein i vävnad från mänsklig prostata. Denna studie kan, om den upprepas i ett större material, ha betydelse för tolkningen av resultatet av PSA-testning av män med misstänkt PCa.

De motsägelsefulla resultaten från studier på sambandet mellan CAG-repetitioner och manliga hormonberoende sjukdomstillstånd kan bero på den statistiska modell som används. Vi använde en modell som förutsättningslöst kan bestämma sambandet vilket är en fördel mot andra metoder då man utgår ifrån ett linjärt samband eller att man delar in männen i grupper. Analysen gjordes på över 500 svenska unga friska män med alla tre statistiska metoder och visade ett samband mellan genotypen och hormoner som styr T regleringen. Samma metoder användes för att undersöka om genotypen kan modifiera effekten av rökning på manliga reproduktionsparametrar, då rökning har visat sig minska spermiekoncentrationen och påverka andra viktiga funktioner som upprätthåller fertiliteten. Vi fann att män som rökte och hade många CAG-repetitioner hade mindre sädesvätska än rökande män med färre CAG -repetitioner, en effekt som inte fanns hos icke-rökare. Våra resultat indikerar att AR-genotypen har en betydelse för känsligheten av miljögifter på den manliga reproduktionsfunktionen.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following persons:

My supervisor, Yvonne Lundberg Giwercman for giving me the opportunity to work with this project and always being there to answer all my questions. I like your enthusiasm about genetics and ability to teach scientific thinking.

My co-supervisors, Lars Rylander and Pirkko Härkönen. Lasse, for patiently helping me explaining the mysterious world of statistics and always having the door open for chatting.

Aleksander Giwercman, for your constructive comments and ability to put my molecular thinking into a bigger perspective. I am impressed by your passion for research and the effort you put into it.

All co-authors, for constructive comments and ideas from different fields of expertise to improve the quality of our manuscripts.

My past and present friends in CRC and RMC, for your willingness to discuss scientific and non-scientific issues. Hannah, for your kindness and all struggle put into our common projects. Hamideh, for every day asking me how I am and is prepared to listen to an honest answer and for helping out in the lab. Paal André, for always cheering us up when coming to CRC. Leon, for making my life easier, not only by helping me with Photoshop and statistics in R but also through telling stories from your time as a PhD student. Thanks to, Kristina, Linus, Kalle, Ida, Magdalena, Yasir, Johannes, Jonatan, Karolina, Sigrid, Anna-Karin, Leif, Mona, Irene, Emeli, Tamae, Nishtman, Patrik, Johan and Oskar. Keep up the good work!

Claes Moreau, Anette Saltin and Heléne Karlsson, for your administrative support.

Mina vänner, tack för att ni finns där även om vi inte ses så ofta som jag önskar. Maria, för du jämt lyssnar och får mig på andra tankar, med dig är Malmö hemma!

Pappa, mamma, Rickard, Sandra och mina underbara syskonbarn, tack för allt stöd, för att ni har gjort mig till den jag är och er kärlek, ni är de bästa!

Carl-Magnus, för att du lugnar ner mig, får mig att le och för din uppmuntran dygnet runt.

*

The research in this thesis was supported by grants from the Swedish Research Council, Swedish Cancer Foundation, the Research fund and Cancer Research Fund of Malmö University Hospital, and the Gunnar Nilsson Cancer foundation.

REFERENCES

Abel J, Haarmann-Stemmann T. An introduction to the molecular basics of aryl hydrocarbon receptor biology. *Biol Chem*. 2010;391:1235-48.

Ackerman CM, Lowe LP, Lee H, Hayes MG, Dyer AR, Metzger BE, Lowe WL, Urbanek M. Ethnic variation in allele distribution of the androgen receptor (AR) (CAG)n repeat. *J Androl*. 2012;33:210-5.

Agency for Toxic Substances & Disease Registry. Toxicological profile for Polycyclic Aromatic Hydrocarbons. U.S. Department of Health & Human Services, Atlanta, USA, 1990.

Ahrens-Fath I, Politz O, Geserick C, Haendler B. Androgen receptor function is modulated by the tissue-specific AR45 variant. *FEBS J*. 2005;272:74-84.

Akinloye O, Gromoll J, Simoni M. Variation in CAG and GGN repeat lengths and CAG/GGN haplotype in androgen receptor gene polymorphism and prostate carcinoma in Nigerians. *Br J Biomed Sci*. 2011;68:138-42.

Akre O, Richiardi L. Does a testicular dysgenesis syndrome exist? *Hum Reprod*. 2009;24:2053-60.

Albertelli MA, O'Mahony OA, Brogley M, Tosoian J, Steinkamp M, Daignault S, Wojno K, Robins DM. Glutamine tract length of human androgen receptors affects hormone-dependent and -independent prostate cancer in mice. *Hum Mol Genet*. 2008;17:98-110.

Albertelli MA, Scheller A, Brogley M, Robins DM. Replacing the mouse androgen receptor with human alleles demonstrates glutamine tract length-dependent effects on physiology and tumorigenesis in mice. *Mol Endocrinol*. 2006;20:1248-60.

Alexander DL, Ganem LG, Fernandez-Salguero P, Gonzalez F, Jefcoate CR. Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis. *J Cell Sci*. 1998;111 (Pt 22):3311-22.

Andersen AG, Jensen TK, Carlsen E, Jorgensen N, Andersson AM, Krarup T, Keiding N, Skakkebaek NE. High frequency of sub-optimal semen quality in an unselected population of young men. *Hum Reprod*. 2000;15:366-72.

Andreotti G, Koutros S, Berndt SI, Hughes Barry K, Hou L, Hoppin JA, Sandler DP, Lubin JH, Burdette LA, Yuenger J *et al.* The Interaction between Pesticide Use and Genetic Variants Involved in Lipid Metabolism on Prostate Cancer Risk. *J Cancer Epidemiol*. 2012;2012:358076.

Andriole GL, Crawford ED, Grubb RL, 3rd, Buys SS, Chia D, Church TR, Fouad MN, Gelmann EP, Kvale PA, Reding DJ *et al.* Mortality results from a randomized prostate-cancer screening trial. *N Engl J Med*. 2009;360:1310-9.

Aneck-Hahn NH, Schulenburg GW, Bornman MS, Farias P, de Jager C. Impaired semen quality associated with environmental DDT exposure in young men living

in a malaria area in the Limpopo Province, South Africa. *J Androl*. 2007;28:423-34.

Anway MD, Memon MA, Uzumcu M, Skinner MK. Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. *J Androl*. 2006;27:868-79.

Asatiani K, von Eckardstein S, Simoni M, Gromoll J, Nieschlag E. CAG repeat length in the androgen receptor gene affects the risk of male infertility. *Int J Androl*. 2003;26:255-61.

Aschim EL, Nordenskjold A, Giwercman A, Lundin KB, Ruhayel Y, Haugen TB, Grotmol T, Giwercman YL. Linkage between cryptorchidism, hypospadias, and GGN repeat length in the androgen receptor gene. *J Clin Endocrinol Metab*. 2004;89:5105-9.

Askew EB, Gampe RT, Jr., Stanley TB, Faggart JL, Wilson EM. Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. *J Biol Chem*. 2007;282:25801-16.

Avances C, Georget V, Terouanne B, Orio F, Cussenot O, Mottet N, Costa P, Sultan C. Human prostatic cell line PNT1A, a useful tool for studying androgen receptor transcriptional activity and its differential subnuclear localization in the presence of androgens and antiandrogens. *Mol Cell Endocrinol*. 2001;184:13-24.

Axelsson J, Rylander L, Rignell-Hydbom A, Giwercman A. No secular trend over the last decade in sperm counts among Swedish men from the general population. *Hum Reprod*. 2011;26:1012-6.

Barnes-Ellerbe S, Knudsen KE, Puga A. 2,3,7,8-Tetrachlorodibenzo-p-dioxin blocks androgen-dependent cell proliferation of LNCaP cells through modulation of pRB phosphorylation. *Mol Pharmacol*. 2004;66:502-11.

Beilin J, Ball EM, Favaloro JM, Zajac JD. Effect of the androgen receptor CAG repeat polymorphism on transcriptional activity: specificity in prostate and non-prostate cell lines. *J Mol Endocrinol*. 2000;25:85-96.

Bennett CL, Price DK, Kim S, Liu D, Jovanovic BD, Nathan D, Johnson ME, Montgomery JS, Cude K, Brockbank JC *et al.* Racial variation in CAG repeat lengths within the androgen receptor gene among prostate cancer patients of lower socioeconomic status. *J Clin Oncol*. 2002;20:3599-604.

Bennett NC, Gardiner RA, Hooper JD, Johnson DW, Gobe GC. Molecular cell biology of androgen receptor signalling. *Int J Biochem Cell Biol*. 2010;42:813-27.

Birkhoj M, Nellemann C, Jarfelt K, Jacobsen H, Andersen HR, Dalgaard M, Vinggaard AM. The combined antiandrogenic effects of five commonly used pesticides. *Toxicol Appl Pharmacol*. 2004;201:10-20.

Bonefeld-Jorgensen EC, Andersen HR, Rasmussen TH, Vinggaard AM. Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. *Toxicology*. 2001;158:141-53.

Bratt O, Borg A, Kristoffersson U, Lundgren R, Zhang QX, Olsson H. CAG repeat length in the androgen receptor gene is related to age at diagnosis of prostate

cancer and response to endocrine therapy, but not to prostate cancer risk. *Br J Cancer*. 1999;81:672-6.

Brinkmann AO, Blok LJ, de Ruiter PE, Doesburg P, Steketee K, Berrevoets CA, Trapman J. Mechanisms of androgen receptor activation and function. *J Steroid Biochem Mol Biol*. 1999;69:307-13.

Brockschmidt FF, Nothen MM, Hillmer AM. The two most common alleles of the coding GGN repeat in the androgen receptor gene cause differences in protein function. *J Mol Endocrinol*. 2007;39:1-8.

Brokken LJ, Lundberg-Giwercman Y, De-Meyts ER, Eberhard J, Stahl O, Cohn-Cedermark G, Daugaard G, Arver S, Giwercman A. Association of polymorphisms in genes encoding hormone receptors ESR1, ESR2 and LHCGR with the risk and clinical features of testicular germ cell cancer. *Mol Cell Endocrinol*. 2012;351:279-85.

Buchanan G, Need EF, Barrett JM, Bianco-Miotto T, Thompson VC, Butler LM, Marshall VR, Tilley WD, Coetzee GA. Corepressor effect on androgen receptor activity varies with the length of the CAG encoded polyglutamine repeat and is dependent on receptor/corepressor ratio in prostate cancer cells. *Mol Cell Endocrinol*. 2011;342:20-31.

Buchanan G, Yang M, Cheong A, Harris JM, Irvine RA, Lambert PF, Moore NL, Raynor M, Neufing PJ, Coetzee GA *et al.* Structural and functional consequences of glutamine tract variation in the androgen receptor. *Hum Mol Genet*. 2004;13:1677-92.

Butland SL, Devon RS, Huang Y, Mead CL, Meynert AM, Neal SJ, Lee SS, Wilkinson A, Yang GS, Yuen MM *et al.* CAG-encoded polyglutamine length polymorphism in the human genome. *BMC Genomics*. 2007;8:126.

Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. *BMJ*. 1992;305:609-13.

Carreau S, Bouraima-Lelong H, Delalande C. Estrogen, a female hormone involved in spermatogenesis. *Adv Med Sci*. 2012;57:31-6.

Castro-Nallar E, Bacallao K, Parada-Bustamante A, Lardone MC, Lopez PV, Madariaga M, Valdevenito R, Piottante A, Ebensperger M, Castro A. Androgen receptor gene CAG and GGN repeat polymorphisms in Chilean men with primary severe spermatogenic failure. *J Androl*. 2010;31:552-9.

Center MM, Jemal A, Lortet-Tieulent J, Ward E, Ferlay J, Brawley O, Bray F. International variation in prostate cancer incidence and mortality rates. *Eur Urol*. 2012;61:1079-92.

Chamberlain NL, Driver ED, Miesfeld RL. The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. *Nucleic Acids Res*. 1994;22:3181-6.

Chamie K, DeVere White RW, Lee D, Ok JH, Ellison LM. Agent Orange exposure, Vietnam War veterans, and the risk of prostate cancer. *Cancer*. 2008;113:2464-70.

Chang CS, Kokontis J, Liao ST. Molecular cloning of human and rat complementary DNA encoding androgen receptors. *Science*. 1988;240:324-6.

Choong CS, Kemppainen JA, Wilson EM. Evolution of the primate androgen receptor: a structural basis for disease. *J Mol Evol*. 1998;47:334-42.

Christiansen S, Scholze M, Dalgaard M, Vinggaard A-M, Axelstad M, Kortenkamp A, Hass U. Synergistic Disruption of External Male Sex Organ Development by a Mixture of Four Antiandrogens. *Environ Health Perspect*. 2009;117:1839-46.

Crabbe P, Bogaert V, De Bacquer D, Goemaere S, Zmierczak H, Kaufman JM. Part of the interindividual variation in serum testosterone levels in healthy men reflects differences in androgen sensitivity and feedback set point: contribution of the androgen receptor polyglutamine tract polymorphism. *J Clin Endocrinol Metab*. 2007;92:3604-10.

Davis-Dao C, Koh CJ, Hardy BE, Chang A, Kim SS, De Filippo R, Hwang A, Pike MC, Carroll JD, Coetzee GA *et al.* Shorter androgen receptor CAG repeat lengths associated with cryptorchidism risk among Hispanic white boys. *J Clin Endocrinol Metab*. 2012;97:E393-9.

Davis-Dao CA, Tuazon ED, Sokol RZ, Cortessis VK. Male infertility and variation in CAG repeat length in the androgen receptor gene: a meta-analysis. *J Clin Endocrinol Metab*. 2007;92:4319-26.

De Jager C, Farias P, Barraza-Villarreal A, Avila MH, Ayotte P, Dewailly E, Dombrowski C, Rousseau F, Sanchez VD, Bailey JL. Reduced seminal parameters associated with environmental DDT exposure and p,p'-DDE concentrations in men in Chiapas, Mexico: a cross-sectional study. *J Androl*. 2006;27:16-27.

Dechanet C, Anahory T, Mathieu Daude JC, Quantin X, Reyftmann L, Hamamah S, Hedon B, Dechaud H. Effects of cigarette smoking on reproduction. *Hum Reprod Update*. 2011;17:76-95.

del Rio Gomez I, Marshall T, Tsai P, Shao YS, Guo YL. Number of boys born to men exposed to polychlorinated byphenyls. *Lancet*. 2002;360:143-4.

Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol*. 2003;43:309-34.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev*. 2009;30:293-342.

Dieckmann KP, Pichlmeier U. Clinical epidemiology of testicular germ cell tumors. *World J Urol*. 2004;22:2-14.

Dietrich C, Kaina B. The aryl hydrocarbon receptor (AhR) in the regulation of cell-cell contact and tumor growth. *Carcinogenesis*. 2010;31:1319-28.

Ding D, Xu L, Menon M, Reddy GP, Barrack ER. Effect of a short CAG (glutamine) repeat on human androgen receptor function. *Prostate*. 2004;58:23-32.

Dowsing AT, Yong EL, Clark M, McLachlan RI, de Kretser DM, Trounson AO. Linkage between male infertility and trinucleotide repeat expansion in the androgen-receptor gene. *Lancet*. 1999;354:640-3.

Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R. Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. *Genomics*. 1992;12:241-53.

Edwards TM, Moore BC, Guillette LJ, Jr. Reproductive dysgenesis in wildlife: a comparative view. *Int J Androl*. 2006;29:109-21.

Engholm G, Ferlay J, Christensen N, Bray F, Gjerstorff ML, Klint A, Kotlum JE, Olafsdottir E, Pukkala E, Storm HH. NORDCAN--a Nordic tool for cancer information, planning, quality control and research. *Acta Oncol*. 2010;49:725-36.

Faber PW, van Rooij HC, Schipper HJ, Brinkmann AO, Trapman J. Two different, overlapping pathways of transcription initiation are active on the TATA-less human androgen receptor promoter. The role of Sp1. *J Biol Chem*. 1993;268:9296-301.

Farr A, Roman A. A pitfall of using a second plasmid to determine transfection efficiency. *Nucleic Acids Res*. 1992 20:1.

Ferlin A, Bartoloni L, Rizzo G, Roverato A, Garolla A, Foresta C. Androgen receptor gene CAG and GGC repeat lengths in idiopathic male infertility. *Mol Hum Reprod*. 2004;10:417-21.

Ferlin A, Garolla A, Bettella A, Bartoloni L, Vinanzi C, Roverato A, Foresta C. Androgen receptor gene CAG and GGC repeat lengths in cryptorchidism. *Eur J Endocrinol*. 2005;152:419-25.

Fietz D, Geyer J, Kliesch S, Gromoll J, Bergmann M. Evaluation of CAG repeat length of androgen receptor expressing cells in human testes showing different pictures of spermatogenic impairment. *Histochem Cell Biol*. 2011;136:689-97.

Fisch H. Declining worldwide sperm counts: disproving a myth. *Urol Clin North Am*. 2008;35:137-46, vii.

Fisch H, Hyun G, Hensle TW. Rising hypospadias rates: disproving a myth. *J Pediatr Urol*. 2010;6:37-9.

Freedman LP, Luisi BF, Korszun ZR, Basavappa R, Sigler PB, Yamamoto KR. The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature*. 1988;334:543-6.

Freedman ML, Pearce CL, Penney KL, Hirschhorn JN, Kolonel LN, Henderson BE, Altshuler D. Systematic evaluation of genetic variation at the androgen receptor locus and risk of prostate cancer in a multiethnic cohort study. *Am J Hum Genet*. 2005;76:82-90.

Friborg J, Koch A, Wohlfarht J, Storm HH, Melbye M. Cancer in Greenlandic Inuit 1973-1997. *Int J Circumpolar Health*. 2004;63 Suppl 2:195-8.

Fritz WA, Lin TM, Cardiff RD, Peterson RE. The aryl hydrocarbon receptor inhibits prostate carcinogenesis in TRAMP mice. *Carcinogenesis*. 2007;28:497-505.

Fritz WA, Lin TM, Safe S, Moore RW, Peterson RE. The selective aryl hydrocarbon receptor modulator 6-methyl-1,3,8-trichlorodibenzofuran inhibits prostate tumor metastasis in TRAMP mice. *Biochem Pharmacol*. 2009;77:1151-60.

Gao T, Marcelli M, McPhaul MJ. Transcriptional activation and transient expression of the human androgen receptor. *J Steroid Biochem Mol Biol*. 1996;59:9-20.

Garolla A, Ferlin A, Vinanzi C, Roverato A, Sotti G, Artibani W, Foresta C. Molecular analysis of the androgen receptor gene in testicular cancer. *Endocr Relat Cancer*. 2005;12:645-55.

Gelmann EP. Molecular biology of the androgen receptor. *J Clin Oncol*. 2002;20:3001-15.

Gioeli D, Paschal BM. Post-translational modification of the androgen receptor. *Mol Cell Endocrinol*. 2012;352:70-8.

Giovannucci E, Stampfer MJ, Krithivas K, Brown M, Dahl D, Brufsky A, Talcott J, Hennekens CH, Kantoff PW. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. *Proc Natl Acad Sci U S A*. 1997;94:3320-3.

Giwercman A, Lundin KB, Eberhard J, Stahl O, Cwikiel M, Cavallin-Stahl E, Giwercman YL. Linkage between androgen receptor gene CAG trinucleotide repeat length and testicular germ cell cancer histological type and clinical stage. *Eur J Cancer*. 2004a;40:2152-8.

Giwercman A, Rylander L, Rignell-Hydbom A, Jonsson BA, Pedersen HS, Ludwicki JK, Lesovoy V, Zvyezday V, Spano M, Manicardi GC *et al.* Androgen receptor gene CAG repeat length as a modifier of the association between persistent organohalogen pollutant exposure markers and semen characteristics. *Pharmacogenet Genomics*. 2007;17:391-401.

Giwercman C, Giwercman A, Pedersen HS, Toft G, Lundin K, Bonde JP, Lundberg Giwercman Y. Polymorphisms in genes regulating androgen activity among prostate cancer low-risk Inuit men and high-risk Scandinavians. *Int J Androl*. 2008;31:25-30.

Giwercman YL, Kleist KE, Giwercman A, Giwercman C, Toft G, Bonde JP, Pedersen HS. Remarkably low incidence of hypospadias in Greenland despite high exposure to endocrine disrupters; possible protective effect of androgen receptor genotype. *Pharmacogenet Genomics*. 2006;16:375-7.

Giwercman YL, Richthoff J, Lilja H, Anderberg C, Abrahamsson PA, Giwercman A. Androgen receptor CAG repeat length correlates with semen PSA levels in adolescence. *Prostate*. 2004b;59:227-33.

Giwercman YL, Xu C, Arver S, Pousette A, Reneland R. No association between the androgen receptor gene CAG repeat and impaired sperm production in Swedish men. *Clin Genet*. 1998;54:435-6.

Goncharov A, Rej R, Negoita S, Schymura M, Santiago-Rivera A, Morse G, Carpenter DO. Lower serum testosterone associated with elevated polychlorinated biphenyl concentrations in Native American men. *Environ Health Perspect*. 2009;117:1454-60.

Gonzalez FJ, Fernandez-Salguero P, Lee SS, Pineau T, Ward JM. Xenobiotic receptor knockout mice. *Toxicol Lett*. 1995;82-83:117-21.

Gottlieb B, Beitel LK, Nadarajah A, Paliouras M, Trifiro M. The androgen receptor gene mutations database: 2012 update. *Hum Mutat*. 2012;33:887-94.

Govarts E, Nieuwenhuijsen M, Schoeters G, Ballester F, Bloemen K, de Boer M, Chevrier C, Eggesbo M, Guxens M, Kramer U *et al.* Birth weight and prenatal exposure to polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethylene (DDE): a meta-analysis within 12 European Birth Cohorts. *Environ Health Perspect*. 2012;120:162-70.

Gray LE, Jr., Kelce WR, Monosson E, Ostby JS, Birnbaum LS. Exposure to TCDD during development permanently alters reproductive function in male Long Evans rats and hamsters: reduced ejaculated and epididymal sperm numbers and sex accessory gland weights in offspring with normal androgenic status. *Toxicol Appl Pharmacol*. 1995;131:108-18.

Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A *et al.* Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum Reprod Update*. 2001;7:248-64.

Griekspoor A, Zwart W, Neefjes J, Michalides R. Visualizing the action of steroid hormone receptors in living cells. *Nucl Recept Signal*. 2007;5:e003.

Grimvall E, Rylander L, Nilsson-Ehle P, Nilsson U, Stromberg U, Hagmar L, Ostman C. Monitoring of polychlorinated biphenyls in human blood plasma: methodological developments and influence of age, lactation, and fish consumption. *Arch Environ Contam Toxicol*. 1997;32:329-36.

Grino PB, Griffin JE, Wilson JD. Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. *Endocrinology*. 1990;126:1165-72.

Gu M, Dong X, Zhang X, Niu W. The CAG repeat polymorphism of androgen receptor gene and prostate cancer: a meta-analysis. *Mol Biol Rep*. 2012;39:2615-24.

Guo YL, Hsu PC, Hsu CC, Lambert GH. Semen quality after prenatal exposure to polychlorinated biphenyls and dibenzofurans. *Lancet*. 2000;356:1240-1.

Guo Z, Qiu Y. A new trick of an old molecule: androgen receptor splice variants taking the stage?! *Int J Biol Sci*. 2011;7:815-22.

Gupta A, Ketchum N, Roehrborn CG, Schecter A, Aragaki CC, Michalek JE. Serum dioxin, testosterone, and subsequent risk of benign prostatic hyperplasia: a prospective cohort study of Air Force veterans. *Environ Health Perspect*. 2006;114:1649-54.

Halmenschlager G, Rossetto S, Lara GM, Rhoden EL. Evaluation of the effects of cigarette smoking on testosterone levels in adult men. *J Sex Med*. 2009;6:1763-72.

Hameed S, Jayasena CN, Dhillo WS. Kisspeptin and fertility. *J Endocrinol*. 2011;208:97-105.

Hardell L, van Bavel B, Lindstrom G, Carlberg M, Dreifaldt AC, Wijkstrom H, Starkhammar H, Eriksson M, Hallquist A, Kolmert T. Increased concentrations of polychlorinated biphenyls, hexachlorobenzene, and chlordanes in mothers of men with testicular cancer. *Environ Health Perspect*. 2003;111:930-4.

Harper PA, Riddick DS, Okey AB. Regulating the regulator: factors that control levels and activity of the aryl hydrocarbon receptor. *Biochem Pharmacol*. 2006;72:267-79.

Hass U, Boberg J, Christiansen S, Jacobsen PR, Vinggaard AM, Taxvig C, Poulsen ME, Herrmann SS, Jensen BH, Petersen A *et al.* Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. *Reprod Toxicol*. 2012;34:261-74.

Hauser R, Chen Z, Pothier L, Ryan L, Altshul L. The relationship between human semen parameters and environmental exposure to polychlorinated biphenyls and p,p'-DDE. *Environ Health Perspect*. 2003;111:1505-11.

Heemers HV, Tindall DJ. Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. *Endocr Rev*. 2007;28:778-808.

Heinlein CA, Chang C. The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Mol Endocrinol*. 2002;16:2181-7.

Holl K, Lundin E, Surcel HM, Grankvist K, Koskela P, Dillner J, Hallmans G, Wadell G, Olafsdottir GH, Ogmundsdottir HM *et al.* Endogenous steroid hormone levels in early pregnancy and risk of testicular cancer in the offspring: a nested case-referent study. *Int J Cancer*. 2009;124:2923-8.

Huhtaniemi IT, Pye SR, Limer KL, Thomson W, O'Neill TW, Platt H, Payne D, John SL, Jiang M, Boonen S *et al.* Increased estrogen rather than decreased androgen action is associated with longer androgen receptor CAG repeats. *J Clin Endocrinol Metab*. 2009;94:277-84.

Inyang F, Ramesh A, Kopsombut P, Niaz MS, Hood DB, Nyanda AM, Archibong AE. Disruption of testicular steroidogenesis and epididymal function by inhaled benzo(a)pyrene. *Reprod Toxicol*. 2003;17:527-37.

Jaaskelainen J. Molecular biology of androgen insensitivity. *Mol Cell Endocrinol*. 2012;352:4-12.

Jana NR, Sarkar S, Ishizuka M, Yonemoto J, Tohyama C, Sone H. Cross-talk between 2,3,7,8-tetrachlorodibenzo-p-dioxin and testosterone signal transduction pathways in LNCaP prostate cancer cells. *Biochem Biophys Res Commun*. 1999;256:462-8.

Janosek J, Hilscherova K, Blaha L, Holoubek I. Environmental xenobiotics and nuclear receptors--interactions, effects and in vitro assessment. *Toxicol In Vitro*. 2006;20:18-37.

Jarow JP, Zirkin BR. The androgen microenvironment of the human testis and hormonal control of spermatogenesis. *Ann N Y Acad Sci*. 2005;1061:208-20.

Jensen TK, Jorgensen N, Punab M, Haugen TB, Suominen J, Zilaitiene B, Horte A, Andersen AG, Carlsen E, Magnus O *et al.* Association of in utero exposure to maternal smoking with reduced semen quality and testis size in adulthood: a cross-sectional study of 1,770 young men from the general population in five European countries. *Am J Epidemiol*. 2004;159:49-58.

Jenster G, van der Korput HA, Trapman J, Brinkmann AO. Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. *J Biol Chem*. 1995;270:7341-6.

Jorgensen N, Carlsen E, Nermoen I, Punab M, Suominen J, Andersen AG, Andersson AM, Haugen TB, Horte A, Jensen TK *et al.* East-West gradient in semen quality in the Nordic-Baltic area: a study of men from the general population in Denmark, Norway, Estonia and Finland. *Hum Reprod*. 2002;17:2199-208.

Jorgensen N, Joensen UN, Jensen TK, Jensen MB, Almstrup K, Olesen IA, Juul A, Andersson AM, Carlsen E, Petersen JH *et al.* Human semen quality in the new millennium: a prospective cross-sectional population-based study of 4867 men. *BMJ Open*. 2012;2.

Kashimada K, Koopman P. Sry: the master switch in mammalian sex determination. *Development*. 2010;137:3921-30.

Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, Lucier G, Luster M, Mac MJ, Maczka C *et al.* Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. *Environ Health Perspect*. 1996;104 Suppl 4:715-40.

Kazemi-Esfarjani P, Trifiro MA, Pinsky L. Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: possible pathogenetic relevance for the (CAG)n-expanded neuronopathies. *Hum Mol Genet*. 1995;4:523-7.

Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, Wilson EM. Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature*. 1995;375:581-5.

Kemppainen JA, Lane MV, Sar M, Wilson EM. Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones. *J Biol Chem*. 1992;267:968-74.

Kittles RA, Young D, Weinrich S, Hudson J, Argyropoulos G, Ukoli F, Adams-Campbell L, Dunston GM. Extent of linkage disequilibrium between the androgen receptor gene CAG and GGC repeats in human populations: implications for prostate cancer risk. *Hum Genet*. 2001;109:253-61.

Kizu R, Okamura K, Toriba A, Kakishima H, Mizokami A, Burnstein KL, Hayakawa K. A role of aryl hydrocarbon receptor in the antiandrogenic effects of polycyclic aromatic hydrocarbons in LNCaP human prostate carcinoma cells. *Arch Toxicol*. 2003;77:335-43.

Kjaerstad MB, Taxvig C, Andersen HR, Nellemann C. Mixture effects of endocrine disrupting compounds in vitro. *Int J Androl*. 2010;33:425-33.

Kollara A, Brown TJ. Functional interaction of nuclear receptor coactivator 4 with aryl hydrocarbon receptor. *Biochem Biophys Res Commun*. 2006;346:526-34.

Kollara A, Brown TJ. Four and a half LIM domain 2 alters the impact of aryl hydrocarbon receptor on androgen receptor transcriptional activity. *J Steroid Biochem Mol Biol*. 2010;118:51-8.

Krausz C. An encore for the repeats: new insights into an old genetic variant. *J Clin Endocrinol Metab*. 2012;97:764-7.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology*. 1998;139:4252-63.

La Spada AR, Roling DB, Harding AE, Warner CL, Spiegel R, Hausmanowa-Petrusewicz I, Yee WC, Fischbeck KH. Meiotic stability and genotype-phenotype correlation of the trinucleotide repeat in X-linked spinal and bulbar muscular atrophy. *Nat Genet*. 1992;2:301-4.

La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature*. 1991;352:77-9.

Lange EM, Sarma AV, Ray A, Wang Y, Ho LA, Anderson SA, Cunningham JM, Cooney KA. The androgen receptor CAG and GGN repeat polymorphisms and prostate cancer susceptibility in African-American men: results from the Flint Men's Health Study. *J Hum Genet*. 2008;53:220-6.

Legius E, Vanderschueren D, Spiessens C, D'Hooghe T, Matthijs G. Association between CAG repeat number in the androgen receptor and male infertility in a Belgian study. *Clin Genet*. 1999;56:166-7.

Lin TM, Ko K, Moore RW, Simanainen U, Oberley TD, Peterson RE. Effects of aryl hydrocarbon receptor null mutation and in utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure on prostate and seminal vesicle development in C57BL/6 mice. *Toxicol Sci*. 2002;68:479-87.

Lindstrom S, Ma J, Altshuler D, Giovannucci E, Riboli E, Albanes D, Allen NE, Berndt SI, Boeing H, Bueno-de-Mesquita HB *et al*. A large study of androgen receptor germline variants and their relation to sex hormone levels and prostate cancer risk. Results from the National Cancer Institute Breast and Prostate Cancer Cohort Consortium. *J Clin Endocrinol Metab*. 2010;95:E121-7.

Llobet JM, Domingo JL, Bocio A, Casas C, Teixido A, Muller L. Human exposure to dioxins through the diet in Catalonia, Spain: carcinogenic and non-carcinogenic risk. *Chemosphere*. 2003;50:1193-200.

Longnecker MP, Rogan WJ, Lucier G. The human health effects of DDT (dichlorodiphenyltrichloroethane) and PCBS (polychlorinated biphenyls) and an overview of organochlorines in public health. *Annu Rev Public Health*. 1997;18:211-44.

Lubahn DB, Brown TR, Simental JA, Higgs HN, Migeon CJ, Wilson EM, French FS. Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. *Proc Natl Acad Sci U S A*. 1989;86:9534-8.

Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science*. 1988;240:327-30.

Luccio-Camelo DC, Prins GS. Disruption of androgen receptor signaling in males by environmental chemicals. *J Steroid Biochem Mol Biol*. 2011;127:74-82.

Lundin KB, Giwercman A, Dizeyi N, Giwercman YL. Functional in vitro characterisation of the androgen receptor GGN polymorphism. *Mol Cell Endocrinol*. 2007;264:184-7.

Lundin KB, Giwercman A, Richthoff J, Abrahamsson PA, Giwercman YL. No association between mutations in the human androgen receptor GGN repeat and inter-sex conditions. *Mol Hum Reprod*. 2003;9:375-9.

Lundin KB, Giwercman YL, Rylander L, Hagmar L, Giwercman A. Androgen receptor gene GGN repeat length and reproductive characteristics in young Swedish men. *Eur J Endocrinol*. 2006;155:347-54.

Mably TA, Moore RW, Peterson RE. In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin. 1. Effects on androgenic status. *Toxicol Appl Pharmacol*. 1992;114:97-107.

MacKenzie KM, Angevine DM. Infertility in mice exposed in utero to benzo(a)pyrene. *Biol Reprod*. 1981;24:183-91.

Mamsen LS, Lutterodt MC, Andersen EW, Skouby SO, Sorensen KP, Andersen CY, Byskov AG. Cigarette smoking during early pregnancy reduces the number of embryonic germ and somatic cells. *Hum Reprod*. 2010;25:2755-61.

McEwan IJ. Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain. *Endocr Relat Cancer*. 2004;11:281-93.

McGlynn KA, Trabert B. Adolescent and adult risk factors for testicular cancer. *Nat Rev Urol*. 2012;9:339-49.

Mehta V, Vezina CM. Potential protective mechanisms of aryl hydrocarbon receptor (AHR) signaling in benign prostatic hyperplasia. *Differentiation*. 2011;82:211-9.

Mifsud A, Choon AT, Fang D, Yong EL. Prostate-specific antigen, testosterone, sex-hormone binding globulin and androgen receptor CAG repeat polymorphisms in subfertile and normal men. *Mol Hum Reprod*. 2001a;7:1007-13.

Mifsud A, Sim CK, Boettger-Tong H, Moreira S, Lamb DJ, Lipshultz LI, Yong EL. Trinucleotide (CAG) repeat polymorphisms in the androgen receptor gene: molecular markers of risk for male infertility. *Fertil Steril*. 2001b;75:275-81.

Miller KP, Ramos KS. Impact of cellular metabolism on the biological effects of benzo[a]pyrene and related hydrocarbons. *Drug Metab Rev*. 2001;33:1-35.

Mimura J, Fujii-Kuriyama Y. Functional role of AhR in the expression of toxic effects by TCDD. *Biochim Biophys Acta*. 2003;1619:263-8.

Mitchell S, Abel P, Ware M, Stamp G, Lalani E. Phenotypic and genotypic characterization of commonly used human prostatic cell lines. *BJU Int*. 2000;85:932-44.

Mitra A, Chakraborty B, Mukhopadhay D, Pal M, Mukherjee S, Banerjee S, Chaudhuri K. Effect of smoking on semen quality, FSH, testosterone level, and CAG repeat length in androgen receptor gene of infertile men in an Indian city. *Syst Biol Reprod Med*. 2012.

Mocarelli P, Gerthoux PM, Needham LL, Patterson DG, Jr., Limonta G, Falbo R, Signorini S, Bertona M, Crespi C, Sarto C *et al.* Perinatal exposure to low doses of dioxin can permanently impair human semen quality. *Environ Health Perspect*. 2011;119:713-8.

Morrow D, Qin C, Smith R, 3rd, Safe S. Aryl hydrocarbon receptor-mediated inhibition of LNCaP prostate cancer cell growth and hormone-induced transactivation. *J Steroid Biochem Mol Biol*. 2004;88:27-36.

Mosaad YM, Shahin D, Elkholy AA, Mosbah A, Badawy W. CAG repeat length in androgen receptor gene and male infertility in Egyptian patients. *Andrologia*. 2012;44:26-33.

Mulholland DJ, Cox M, Read J, Rennie P, Nelson C. Androgen responsiveness of Renilla luciferase reporter vectors is promoter, transgene, and cell line dependent. *Prostate*. 2004;59:115-9.

Nenonen HA, Giwercman A, Hallengren E, Giwercman YL. Non-linear association between androgen receptor CAG repeat length and risk of male subfertility--a meta-analysis. *Int J Androl*. 2011;34:327-32.

Nieschlag E, Behre HM. Andrology Male Reproductive Health and Dysfunction. 2 edn. Springer-Verlag, Berlin, Heidelberg 2001.

Niu Y, Altuwaijri S, Lai KP, Wu CT, Ricke WA, Messing EM, Yao J, Yeh S, Chang C. Androgen receptor is a tumor suppressor and proliferator in prostate cancer. *Proc Natl Acad Sci U S A*. 2008;105:12182-7.

Ohtake F, Baba A, Fujii-Kuriyama Y, Kato S. Intrinsic AhR function underlies cross-talk of dioxins with sex hormone signalings. *Biochem Biophys Res Commun*. 2008;370:541-6.

Ohtake F, Baba A, Takada I, Okada M, Iwasaki K, Miki H, Takahashi S, Kouzmenko A, Nohara K, Chiba T *et al.* Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. *Nature*. 2007;446:562-6.

Ohtake F, Fujii-Kuriyama Y, Kato S. AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions. *Biochem Pharmacol*. 2009;77:474-84.

Parada-Bustamante A, Lardone MC, Madariaga M, Johnson MC, Codner E, Cassorla F, Castro A. Androgen receptor CAG and GGN polymorphisms in boys with isolated hypospadias. *J Pediatr Endocrinol Metab*. 2012;25:157-62.

Pavuk M, Michalek JE, Ketchum NS. Prostate cancer in US Air Force veterans of the Vietnam war. *J Expo Sci Environ Epidemiol*. 2006;16:184-90.

Peng X, Zeng X, Peng S, Deng D, Zhang J. The association risk of male subfertility and testicular cancer: a systematic review. *PLoS ONE*. 2009;4:e5591.

Pesatori AC, Consonni D, Rubagotti M, Grillo P, Bertazzi PA. Cancer incidence in the population exposed to dioxin after the "Seveso accident": twenty years of follow-up. *Environ Health*. 2009;8:39.

Platz EA, Giovannucci E, Dahl DM, Krithivas K, Hennekens CH, Brown M, Stampfer MJ, Kantoff PW. The androgen receptor gene GGN microsatellite and prostate cancer risk. *Cancer Epidemiol Biomarkers Prev*. 1998;7:379-84.

Powell IJ, Land SJ, Dey J, Heilbrun LK, Hughes MR, Sakr W, Everson RB. The impact of CAG repeats in exon 1 of the androgen receptor on disease progression after prostatectomy. *Cancer*. 2005;103:528-37.

Prince MM, Ruder AM, Hein MJ, Waters MA, Whelan EA, Nilsen N, Ward EM, Schnorr TM, Laber PA, Davis-King KE. Mortality and exposure response among 14,458 electrical capacitor manufacturing workers exposed to polychlorinated biphenyls (PCBs). *Environ Health Perspect*. 2006;114:1508-14.

Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS. Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev*. 1995;16:271-321.

R Development Core Team. R: A language and environment for statistical computing, Vienna, Austria 2012.

Radpour R, Rezaee M, Tavasoly A, Solati S, Saleki A. Association of long polyglycine tracts (GGN repeats) in exon 1 of the androgen receptor gene with cryptorchidism and penile hypospadias in Iranian patients. *J Androl*. 2007;28:164-9.

Rajpert-De Meyts E, Leffers H, Daugaard G, Andersen CB, Petersen PM, Hinrichsen J, Pedersen LG, Skakkebaek NE. Analysis of the polymorphic CAG repeat length in the androgen receptor gene in patients with testicular germ cell cancer. *Int J Cancer*. 2002a;102:201-4.

Rajpert-De Meyts E, Leffers H, Petersen JH, Andersen AG, Carlsen E, Jorgensen N, Skakkebaek NE. CAG repeat length in androgen-receptor gene and reproductive variables in fertile and infertile men. *Lancet*. 2002b;359:44-6.

Ramlau-Hansen CH, Thulstrup AM, Aggerholm AS, Jensen MS, Toft G, Bonde JP. Is smoking a risk factor for decreased semen quality? A cross-sectional analysis. *Hum Reprod*. 2007a;22:188-96.

Ramlau-Hansen CH, Thulstrup AM, Storgaard L, Toft G, Olsen J, Bonde JP. Is prenatal exposure to tobacco smoking a cause of poor semen quality? A follow-up study. *Am J Epidemiol*. 2007b;165:1372-9.

Ravnborg TL, Jensen TK, Andersson AM, Toppari J, Skakkebaek NE, Jorgensen N. Prenatal and adult exposures to smoking are associated with adverse effects on reproductive hormones, semen quality, final height and body mass index. *Hum Reprod*. 2011;26:1000-11.

Raychoudhury SS, Kubinski D. Polycyclic aromatic hydrocarbon-induced cytotoxicity in cultured rat Sertoli cells involves differential apoptotic response. *Environ Health Perspect*. 2003;111:33-8.

Rey RA, Grinspon RP. Normal male sexual differentiation and aetiology of disorders of sex development. *Best Pract Res Clin Endocrinol Metab*. 2011;25:221-38.

Rey RA, Musse M, Venara M, Chemes HE. Ontogeny of the androgen receptor expression in the fetal and postnatal testis: its relevance on Sertoli cell maturation and the onset of adult spermatogenesis. *Microsc Res Tech*. 2009;72:787-95.

Reyes-Hernandez OD, Mejia-Garcia A, Sanchez-Ocampo EM, Castro-Munozledo F, Hernandez-Munoz R, Elizondo G. Aromatic hydrocarbons upregulate glyceraldehyde-3-phosphate dehydrogenase and induce changes in actin cytoskeleton. Role of the aryl hydrocarbon receptor (AhR). *Toxicology*. 2009;266:30-7.

Richiardi L, Bellocco R, Adami HO, Torrang A, Barlow L, Hakulinen T, Rahu M, Stengrevics A, Storm H, Tretli S *et al.* Testicular cancer incidence in eight northern European countries: secular and recent trends. *Cancer Epidemiol Biomarkers Prev*. 2004;13:2157-66.

Richthoff J, Elzanaty S, Rylander L, Hagmar L, Giwercman A. Association between tobacco exposure and reproductive parameters in adolescent males. *Int J Androl*. 2008;31:31-9.

Rider CV, Furr J, Wilson VS, Gray LE, Jr. A mixture of seven antiandrogens induces reproductive malformations in rats. *Int J Androl*. 2008;31:249-62.

Rignell-Hydbom A, Rylander L, Giwercman A, Jonsson BA, Nilsson-Ehle P, Hagmar L. Exposure to CB-153 and p,p'-DDE and male reproductive function. *Hum Reprod*. 2004;19:2066-75.

Robins DM, Albertelli MA, O'Mahony OA. Androgen receptor variants and prostate cancer in humanized AR mice. *J Steroid Biochem Mol Biol*. 2008;108:230-6.

Roddam AW, Allen NE, Appleby P, Key TJ. Endogenous sex hormones and prostate cancer: a collaborative analysis of 18 prospective studies. *J Natl Cancer Inst*. 2008;100:170-83.

Rodriguez-Gonzalez G, Cabrera S, Ramirez-Moreno R, Bilbao C, Diaz-Chico JC, Serra L, Chesa N, Cabrera JJ, Diaz-Chico BN. Short alleles of both GGN and CAG repeats at the exon-1 of the androgen receptor gene are associated to

increased PSA staining and a higher Gleason score in human prostatic cancer. *J Steroid Biochem Mol Biol*. 2009;113:85-91.

Rogol AD. Androgens and puberty. *Mol Cell Endocrinol*. 2002;198:25-9.

Roy P, Salminen H, Koskimies P, Simola J, Smeds A, Saukko P, Huhtaniemi IT. Screening of some anti-androgenic endocrine disruptors using a recombinant cell-based in vitro bioassay. *J Steroid Biochem Mol Biol*. 2004;88:157-66.

Salinas CA, Austin MA, Ostrander EO, Stanford JL. Polymorphisms in the androgen receptor and the prostate-specific antigen genes and prostate cancer risk. *Prostate*. 2005;65:58-65.

Sanada N, Gotoh Y, Shimazawa R, Klinge CM, Kizu R. Repression of activated aryl hydrocarbon receptor-induced transcriptional activation by 5alpha-dihydrotestosterone in human prostate cancer LNCaP and human breast cancer T47D cells. *J Pharmacol Sci*. 2009;109:380-7.

Schecter A, Birnbaum L, Ryan JJ, Constable JD. Dioxins: an overview. *Environ Res*. 2006;101:419-28.

Schell LM, Burnitz KK, Lathrop PW. Pollution and human biology. *Ann Hum Biol*. 2010;37:347-66.

Schnack TH, Poulsen G, Myrup C, Wohlfahrt J, Melbye M. Familial coaggregation of cryptorchidism, hypospadias, and testicular germ cell cancer: a nationwide cohort study. *J Natl Cancer Inst*. 2010;102:187-92.

Schrader TJ, Cooke GM. Effects of Aroclors and individual PCB congeners on activation of the human androgen receptor in vitro. *Reprod Toxicol*. 2003;17:15-23.

Schroder FH, Hugosson J, Roobol MJ, Tammela TL, Ciatto S, Nelen V, Kwiatkowski M, Lujan M, Lilja H, Zappa M *et al.* Screening and prostate-cancer mortality in a randomized European study. *N Engl J Med*. 2009;360:1320-8.

Shah SR, Freedland SJ, Aronson WJ, Kane CJ, Presti JC, Jr., Amling CL, Terris MK. Exposure to Agent Orange is a significant predictor of prostate-specific antigen (PSA)-based recurrence and a rapid PSA doubling time after radical prostatectomy. *BJU Int*. 2009;103:1168-72.

Sharpe RM. Environmental/lifestyle effects on spermatogenesis. *Philos Trans R Soc Lond B Biol Sci*. 2010;365:1697-712.

Shen HC, Coetzee GA. The androgen receptor: unlocking the secrets of its unique transactivation domain. *Vitam Horm*. 2005;71:301-19.

Simanainen U, Brogley M, Gao YR, Jimenez M, Harwood DT, Handelsman DJ, Robins DM. Length of the human androgen receptor glutamine tract determines androgen sensitivity in vivo. *Mol Cell Endocrinol*. 2011;342:81-6.

Simanainen U, Haavisto T, Tuomisto JT, Paranko J, Toppari J, Tuomisto J, Peterson RE, Viluksela M. Pattern of male reproductive system effects after in utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure in three differentially TCDD-sensitive rat lines. *Toxicol Sci*. 2004;80:101-8.

Simon S, Mueller SO. Human reporter gene assays: transcriptional activity of the androgen receptor is modulated by the cellular environment and promoter context. *Toxicology*. 2006;220:90-103.

Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod*. 2001;16:972-8.

Soares SR, Melo MA. Cigarette smoking and reproductive function. *Curr Opin Obstet Gynecol*. 2008;20:281-91.

Sofikitis N, Miyagawa I, Dimitriadis D, Zavos P, Sikka S, Hellstrom W. Effects of smoking on testicular function, semen quality and sperm fertilizing capacity. *J Urol*. 1995;154:1030-4.

Sorg O, Zennegg M, Schmid P, Fedosyuk R, Valikhnovskyi R, Gaide O, Kniazevych V, Saurat JH. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) poisoning in Victor Yushchenko: identification and measurement of TCDD metabolites. *Lancet*. 2009;374:1179-85.

Storgaard L, Bonde JP, Ernst E, Spano M, Andersen CY, Frydenberg M, Olsen J. Does smoking during pregnancy affect sons' sperm counts? *Epidemiology*. 2003;14:278-86.

Stukenborg JB, Colon E, Soder O. Ontogenesis of testis development and function in humans. *Sex Dev*. 2010;4:199-212.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S *et al.* Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect*. 2005;113:1056-61.

Svensson BG, Nilsson A, Jonsson E, Schutz A, Akesson B, Hagmar L. Fish consumption and exposure to persistent organochlorine compounds, mercury, selenium and methylamines among Swedish fishermen. *Scand J Work Environ Health*. 1995;21:96-105.

Tiido T, Rignell-Hydbom A, Jonsson BA, Giwercman YL, Pedersen HS, Wojtyniak B, Ludwicki JK, Lesovoy V, Zvyezday V, Spano M *et al.* Impact of PCB and p,p'-DDE contaminants on human sperm Y:X chromosome ratio: studies in three European populations and the Inuit population in Greenland. *Environ Health Perspect*. 2006;114:718-24.

Tijet N, Boutros PC, Moffat ID, Okey AB, Tuomisto J, Pohjanvirta R. Aryl hydrocarbon receptor regulates distinct dioxin-dependent and dioxin-independent gene batteries. *Mol Pharmacol*. 2006;69:140-53.

Tilley WD, Marcelli M, Wilson JD, McPhaul MJ. Characterization and expression of a cDNA encoding the human androgen receptor. *Proc Natl Acad Sci U S A*. 1989;86:327-31.

Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jr., Jegou B, Jensen TK, Jouannet P, Keiding N *et al.* Male reproductive health and

environmental xenoestrogens. *Environ Health Perspect*. 1996;104 Suppl 4:741-803.

Tse JY, Liu VW, Yeung WS, Lau EY, Ng EH, Ho PC. Molecular analysis of the androgen receptor gene in Hong Kong Chinese infertile men. *J Assist Reprod Genet*. 2003;20:227-33.

Tsujimoto Y, Takakuwa T, Takayama H, Nishimura K, Okuyama A, Aozasa K, Nonomura N. In situ shortening of CAG repeat length within the androgen receptor gene in prostatic cancer and its possible precursors. *Prostate*. 2004;58:283-90.

Tut TG, Ghadessy FJ, Trifiro MA, Pinsky L, Yong EL. Long polyglutamine tracts in the androgen receptor are associated with reduced trans-activation, impaired sperm production, and male infertility. *J Clin Endocrinol Metab*. 1997;82:3777-82.

van den Driesche S, Kolovos P, Platts S, Drake AJ, Sharpe RM. Inter-relationship between testicular dysgenesis and Leydig cell function in the masculinization programming window in the rat. *PLoS ONE*. 2012;7:e30111.

Van Maele-Fabry G, Willems JL. Occupation related pesticide exposure and cancer of the prostate: a meta-analysis. *Occup Environ Med*. 2003;60:634-42.

Vastermark A, Giwercman YL, Hagstromer O, De-Meyts ER, Eberhard J, Stahl O, Cedermark GC, Rastkhani H, Daugaard G, Arver S *et al.* Polymorphic variation in the androgen receptor gene: association with risk of testicular germ cell cancer and metastatic disease. *Eur J Cancer*. 2011;47:413-9.

Welsh M, Saunders PT, Sharpe RM. The critical time window for androgen-dependent development of the Wolffian duct in the rat. *Endocrinology*. 2007;148:3185-95.

Verhoeven G, Willems A, Denolet E, Swinnen JV, De Gendt K. Androgens and spermatogenesis: lessons from transgenic mouse models. *Philos Trans R Soc Lond B Biol Sci*. 2010;365:1537-56.

Werner R, Holterhus PM, Binder G, Schwarz HP, Morlot M, Struve D, Marschke C, Hiort O. The A645D mutation in the hinge region of the human androgen receptor (AR) gene modulates AR activity, depending on the context of the polymorphic glutamine and glycine repeats. *J Clin Endocrinol Metab*. 2006;91:3515-20.

Vijayalakshmi K, Thangaraj K, Rajender S, Vettriselvi V, Venkatesan P, Shroff S, Vishwanathan KN, Paul SF. GGN repeat length and GGN/CAG haplotype variations in the androgen receptor gene and prostate cancer risk in south Indian men. *J Hum Genet*. 2006;51:998-1005.

Wilson CM, McPhaul MJ. A and B forms of the androgen receptor are present in human genital skin fibroblasts. *Proc Natl Acad Sci U S A*. 1994;91:1234-8.

Vinggaard AM, Hnida C, Larsen JC. Environmental polycyclic aromatic hydrocarbons affect androgen receptor activation in vitro. *Toxicology*. 2000;145:173-83.

von Eckardstein S, Syska A, Gromoll J, Kamischke A, Simoni M, Nieschlag E. Inverse correlation between sperm concentration and number of androgen receptor CAG repeats in normal men. *J Clin Endocrinol Metab*. 2001;86:2585-90.

Wong C, Kelce WR, Sar M, Wilson EM. Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *J Biol Chem*. 1995;270:19998-20003.

Woodham C, Birch L, Prins GS. Neonatal estrogen down-regulates prostatic androgen receptor through a proteosome-mediated protein degradation pathway. *Endocrinology*. 2003;144:4841-50.

World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm - Cervical Mucus Interaction. 4th edn. Published on the behalf of the World Health Organization [by] Cambridge University Press, Cambridge; UK, 1999.

Yeap BB, Wilce JA, Leedman PJ. The androgen receptor mRNA. *Bioessays*. 2004;26:672-82.

Yoshioka H, Hiromori Y, Aoki A, Kimura T, Fujii-Kuriyama Y, Nagase H, Nakanishi T. Possible aryl hydrocarbon receptor-independent pathway of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced antiproliferative response in human breast cancer cells. *Toxicol Lett*. 2012;211:257-65.

Zafar MB, Terris MK. Prostate cancer detection in veterans with a history of Agent Orange exposure. *J Urol*. 2001;166:100-3.

Zeegers MP, Kiemeney LA, Nieder AM, Ostrer H. How strong is the association between CAG and GGN repeat length polymorphisms in the androgen receptor gene and prostate cancer risk? *Cancer Epidemiol Biomarkers Prev*. 2004;13:1765-71.

Zenzes MT, Bielecki R, Reed TE. Detection of benzo(a)pyrene diol epoxide-DNA adducts in sperm of men exposed to cigarette smoke. *Fertil Steril*. 1999;72:330-5.