ANDROGEN RECEPTOR CAG AND GGN POLYMORPHISMS IN VIVO AND IN VITRO

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List of papers

This thesis is based on studies reported in following papers, which are referred to in the text by their Roman numerals (I-IV):

I Skjærpe PA, Lundberg Giwercman Y, Giwercman A, Svartberg J.

II Skjærpe PA, Lundberg Giwercman Y, Giwercman A, Svartberg J.
Androgen receptor gene polymorphism and the metabolic syndrome in 60-80 years old Norwegian men.

III Nenonen H, Björk C, Skjærpe PA, Giwercman A, Rylander L, Svartberg J, Lundberg Giwercman Y.
CAG repeat number is not inversely associated with androgen receptor activity in vitro.

Androgen receptor CAG length dependent amount of prostate specific antigen in serum and in tissue.
Submitted manuscript.
## Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AIS</td>
<td>Androgen insensitivity syndrome</td>
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<tr>
<td>AR</td>
<td>Androgen receptor</td>
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<tr>
<td>ARA24</td>
<td>Androgen receptor associated protein 24</td>
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<td>ARA70</td>
<td>Androgen receptor associated protein 70</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<td>BPH</td>
<td>Benign prostate hyperplasia</td>
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<td>CAIS</td>
<td>Complete androgen insensitivity syndrome</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>dGTP</td>
<td>Deoxy guanine triphosphate</td>
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<td>DHT</td>
<td>Dihydrotestosterone</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ER</td>
<td>Oestrogen receptor</td>
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<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>HbA1c</td>
<td>Glycosylated haemoglobin</td>
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<td>HDL-C</td>
<td>High-density lipoprotein cholesterol</td>
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<tr>
<td>HPT</td>
<td>Hypothalamic-pituitary-testicular hormone</td>
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<td>LH</td>
<td>Luteinizing hormone</td>
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<tr>
<td>MAIS</td>
<td>Mild androgen insensitivity syndrome</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<td>PAIS</td>
<td>Partial androgen insensitivity syndrome</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>qPCR</td>
<td>Real-time quantitative PCR</td>
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<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
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<tr>
<td>SHBG</td>
<td>Sex hormone binding globulin</td>
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<tr>
<td>SBMA</td>
<td>Spinal and bulbar muscular</td>
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<tr>
<td>WC</td>
<td>Waist circumference</td>
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<td>TRFI</td>
<td>Time resolved fluorescence imaging</td>
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Introduction

The anabolic steroid hormones are of key importance in the integrated control of metabolic homeostasis and sexual differentiation. Since their initial discovery in the 1930s, the androgenic hormones, in particular testosterone and its derivatives have been appreciated as the major factors in controlling the development and maintenance of male sexual characteristics [1].

Androgen functions are mediated by binding to the androgen receptor (AR), which is found to varying extent in a range of tissues [2]. The importance of androgenic hormones in glucose and fatty acid metabolism form the mechanistic basis for many of the gender differences in the risk of developing a range of diseases, including cardiovascular and malignant diseases [3-6].

This has fueled a considerable interest in androgenic hormone abundance as a risk factor for various human pathological conditions as for example androgen insensitivity syndrome, the cluster of metabolic derangements referred to as the metabolic syndrome [7] as well as benign prostate hypertrophy and prostate cancer [6, 8-10].

Given the central role of the AR in mediating downstream effects of androgenic signaling, factors controlling AR abundance and activity play a major role in regulating overall androgen activity. A number of studies have addressed the relationship between AR composition and function, and mutations in the AR gene have major developmental implications [11, 12]. However, there are still a number of fundamental issues pertaining to the relationship between the AR structure and downstream functions that remain elusive. This work was initiated to further investigate some of the functional consequences of two well-recognized polymorphisms within the AR gene; the CAG and GGN repeat sequences.

Androgen regulation and production

Androgens are a group of chemically related sex steroid hormones that stimulate and control the development and maintenance of male characteristics and are regulated by the hypothalamic-pituitary-testicular hormone (HPT) axis, Figure 1. Androgens also affect functions in non-reproductive tissue, such as bone, skeletal muscle and hair growth.
in both male and females. The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are produced and secreted by the gonadotropic cells of the anterior pituitary as a result of the pulsatile secretion of gonadotropin-releasing hormone (GnRH) in the hypothalamus [13]. LH interacts with receptors on the testicular Leydig cell membrane, and regulates the production and secretion of testosterone, while FSH regulates the spermatogenesis [14]. The gonadotropin secretion is controlled by negative feedback between the gonads, pituitary and hypothalamus. Testosterone acts on the hypothalamus by reducing the hypothalamic pulse generator and inhibits the secretion of LH [15], while the main feedback control of FSH secretion is through Inhibin B from the Sertoli cells [16]. Testosterone also appears to inhibit the secretion of GnRH, LH and FSH by the local conversion of testosterone to estradiol [17].

Figure 1. GnRH is synthesized and released from the hypothalamus. At the pituitary, GNRH stimulates the synthesis and secretion of FSH and LH. These processes are controlled by the size and frequency of GnRH pulses, as well as by feedback from androgens and estrogens. Low frequency GnRH pulses lead to FSH release, whereas high frequency GNRH pulses stimulate LH release. (Drawing by Rod Wolstenholme, University of Tromsø 2012)
Testicular androgens are synthesized by the Leydig cells in the interstitial tissue [1]. The immediate precursor of the gonadal steroids, as well as of the adrenal steroids, is cholesterol. The conversion of cholesterol to testosterone requires the action of five enzymes: \(3\beta\)-hydroxysteroid dehydrogenase (\(3\beta\)-HSD), \(\Delta^{5,4}\)-isomerase, \(17\alpha\)-hydroxylase, \(C_{17,20}\) lyase, and \(17\beta\)-hydroxysteroid dehydrogenase (\(17\beta\)-HSD) [18], Figure 2. The rate limiting step, as in the adrenals, is cholesterol side chain cleavage. The conversion of cholesterol to pregnenolone is identical in the adrenals, in the ovaries and in the testes. In the latter 2 tissues, however, the reaction is promoted by LH rather than by adrenocorticotropic hormone (ACTH). Testosterone is metabolized to \(5\alpha\)-dihydrotestosterone (DHT) by \(5\alpha\)-reductase as well as to estradiol by the enzyme CYP19A1 (aromatase) [1, 19, 20].

DHT is the most potent of the male steroid hormones, and is thought to be approximately 30 times more potent than testosterone [21]. Testosterone is present in the circulation as free testosterone (2-3\%) or bound to albumin (~50\%) and to sex hormone binding globulin (SHBG) (~44\%). Free testosterone was long believed to be the main fraction of testosterone available to the target organs and tissue and responsible for the biological activity [22-24]. The binding between testosterone and albumin, however, is easily reversed and together these two fractions are referred to as active testosterone nowadays,
or bio-available testosterone [24, 25]. In target tissues, testosterone dissociates from carrier proteins and enters the cell via passive diffusion [1]. When bound to the intracellular AR in the cytoplasm, removal of inhibitory proteins and activation of the receptor to its DNA binding state takes place. The hormone-receptor complex is transported to the nucleus where it undergoes dimerisation and binding to hormone response elements of target genes. Transcription activation takes place, resulting in a cellular response, Figure 3 [26].

Figure 3. The androgen receptor is maintained in an inactive complex in the cell cytoplasm bound to heat shock proteins, HSP40, HSP70 and HSP90. Upon ligand binding, conformational changes take place and the receptor is phosphorylated. Importin a binds to the nuclear localisation signal in the hinge region and mediates transport into the nucleus where it dimerises and activates a target gene, leading to gene transcription and an androgenic effect.
The androgen receptor

The human AR belongs to the nuclear receptor family of ligand-activated transcription factors [27]. Its regulation of transcription is crucial for androgenic effects in individuals of both genders, and is important in all phases of human life; differentiation of the sexual organs during the embryogenesis and pubertal development with secondary sex characteristics that typify the adult man i.e. growth of facial and pubic hair, deepening of the voice, development of skeletal muscle mass and bone density as well as sperm production [2, 28, 29]. The AR has been detected in a vast array of genital and non-genital tissues, and can be found not only in the classical androgen-dependent organs, such as muscles, prostate, seminal vesicles, epididymis and testes, but also in almost every tissue, e.g., hypothalamus, pituitary, kidney, spleen, heart and salivary glands [2, 30-33]. The classical target cells for DHT are the prostate, seminal vesicles, external genitalia, hair follicles, and genital skin [1, 34]. Targets for testosterone include the embryonic Wolffian structures, spermatogonia, muscles, bone, kidney, and brain, to mention a few [1, 2, 34, 35].

The AR is located on the X-chromosome at Xq11.2–q12 [36, 37], spanning more than 90 kilobases in length and containing eight exons. The molecular weight is approximately 110 kDa [33, 38]. Like all other steroid receptors, the AR encodes a protein with three major functional domains: the DNA-binding domain, the C-terminal ligand-binding domain [37], and the NH2-terminal transactivating domain [39, 40], Figure 4.

The transactivating domain

The transactivating domain, which is an important determinant of the AR’s capacity to influence target genes [33, 41], contains two polymorphic repeats; a stretch of a variable number of the amino acid glutamine- as well as a glycine tracts encoded by \((CAG)_{n}CAA\) and \((GGT)_{3}(GGG)(GGT)_{2}(GGC)_{n}\), respectively [42]. These sequences are commonly referred to as the CAG and the GGN repeats, respectively.

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Figure 4. Human androgen receptor (AR) gene; structural organization and protein. The AR gene was mapped to the long arm on chromosome X. The hAR protein is encoded by 8 exons (numbered within boxes), separated by introns. The AR protein consists of several distinct functional domains: exon 1 encodes the amino terminal domain involved in transcription regulation; exons 2 and 3 encode the DNA-binding domain; the 5’ end of exon 4 encodes the hinge region including the nuclear targeting signal; the 3’ portion of exon 4 and exons 5-8 encode the steroid-binding domain. The diagram of the protein structure illustrates the main functional domains: the transcription-regulation domain the trans-activating domain, the DNA-binding domain, the hinge region and the ligand-binding domain.

The CAG repeat sequence spans from approximately 10 to 30 repeats, with an average length of 22 repeats in Caucasians [43]. Ethnical differences in the CAG repeat length are well known [44], with on average shorter repeats in African populations, longer repeats in Asians, and intermediate lengths in Caucasians. Abnormal expansion of the CAG repeat length (>40CAG) leads to the neuromuscular disease Spinal and bulbar muscular atrophy (SBMA) also known as Kennedy’s disease, which is accompanied by signs of hypoandrogenism such as gynecomastia, infertility and high plasma concentrations of LH, FSH and estradiol [45]. The severity of the neurological features of the disease have been inversely correlated to the extension of the CAG repeats [46]. These findings, together...
with a few in vitro studies [47-49], have supported the hypothesis of an inverse correlation between transcriptional activity and the CAG repeat length. The in vitro studies showed a significant difference in transactivation between ARs containing the shortest and the longest repeat, respectively, with a decreased activity for the longest repeat compared with the shortest. Notably, in most previous studies, extreme CAG lengths were used. In those with CAG numbers within normal range, the longest repeat displayed decreased activity compared to the shortest, but neither the shorter nor the longer repeat differed in capability to activate the reporter gene compared to the intermediate one [47, 49].

Regarding the GGN segment little is known so far both with respect to normal physiological function and possible pathophysiological conditions. In general, the GGN repeats span from 10 to 27, with 23 GGN as the most common variant, carried by approximately 50% of Caucasian populations, closely followed by 24 GGN, which 30% are carriers of [50]. The importance of the GGN stretch for transcriptional function of the AR was first shown through deletion of the entire repeat, which resulted in 30% reduction of the AR activity [51]. More recent in vitro data have indicated that the 23 GGN allele in combination with 22 CAG gives a higher AR activity compared to longer and shorter GGN alleles, respectively [52]. There are no strong associations with any diseases reported, although short GGN repeats have been reported to correlate with decreased semen volume [43], indicative of lower androgen activity. In middle-aged and old men, GGN numbers different from 23 combined with CAG numbers of 23 or less was reported to be associated with a higher waist-to-hip ratio [53]. As with the CAG repeat, the GGN repeat also varies between populations. Whereas the GGN tract is normally distributed in African populations, this is not the case for Caucasians, who have either 23 or 24 repeats [44].

The DNA-binding domain
The central region of the AR, encoded by exon 2 and 3, contains the DNA-binding domain (DBD), which is the most highly conserved region among members of the steroid hormone receptor family, the AR DNA binding motif having approximately 80% amino
acid identity with those of the mineralocorticoid receptor, the progesterone receptor, and the glucocorticoid receptor (GR) [30].

The DBD contains 9 conserved cysteine residues, of which 8 are involved in the formation of 2 zinc clusters, arranged as a pair of loop structures folded to form a single structural unit made up of two zinc-binding motifs [33, 41, 54, 55]. Four cysteine residues, present in all steroid receptors, coordinately bind a zinc atom in a tetrahedral array in each of the two motifs, thus commonly referred to as zinc fingers. The first zinc finger is encoded by exon 2 and the second zinc finger by exon 3. By analogy with other steroid receptors, the DNA binding domain determines the specificity of AR interaction with DNA [41]. The ability to discriminate between response elements resides in 3 amino acids at the base of the first zinc finger (glycine577, serine578 and valine581), located in the so-called proximal box (P-box). These three amino acids interact with transcriptional enhancer nucleotide sequences referred to as hormone response elements, present in or near target genes [56-58]. The second zincfinger stabilizes DNA and AR interaction by contact with the DNA phosphate backbone. In this part of the AR, a five amino acid residue long distal box (D-box) was identified. The D-box is important as it specifies the half-site spacing requisite at the hormone response element and provides a site for homodimerisation of ARs [58, 59].

The hinge region

The DNA binding domain and the ligand binding domain are coupled by a region of low sequence homology among the members of the steroid hormone receptor-family, known as the hinge (approximately amino acids 623-671) [56]. This region is encoded by the 5’ portion of exon 4 and contains the major part of the AR nuclear localization signal, which consists of two basic amino acid residues, a spacer region of ten amino acids and a basic cluster where three of the next five residues must be basic [56]. This bipartite motif is conserved among steroid receptors, and for GR and AR, this signal (arg, lys, leu, lys, lys at positions 629-633) is necessary and sufficient for hormone dependent nuclear translocation [41].
The ligand binding domain

The carboxy-terminal third of the AR, encoded by the 3’ end of exon 4 and exons 5-8 comprises the hormone binding domain [30]. A principal function of the AR steroid binding domain is the specific, high affinity binding of androgens, but apart from binding of hormones, this part of the AR also is involved in dimerisation and in transcription activation [41]. Steroid hormone receptors are bound by a complex of inhibitory proteins, heat-shock proteins, which are critical for proper folding of the receptors, Figure 3. Upon hormone binding, removal of heat-shock proteins, such as hsp90, may unmask functional domains and initiate conformational changes necessary for nuclear import of the receptor-hormone complex, dimerisation, and DNA binding [33, 41, 60].

Androgen related pathological conditions in males

The androgen insensitivity syndrome.

Androgen insensitivity syndrome (AIS) is a rare disorder of male sexual differentiation caused by an absent or dysfunctional AR [61]. It is an X-linked recessive disorder, only affecting individuals having a 46,XY karyotype. The phenotype is encompassing a wide array of genital ambiguities, which may range from completely female to fertile male without complains of undermasculinization [62]. Generally, normal but immature testes are present and as differentiation of the embryonic Wolffian ducts occur in response to androgens, Wolffian ducts are absent in individuals with AIS. Müllerian ducts are usually absent, as the Anti-Müllerian hormone action in the fetus is normal. The syndrome is classified into three forms: complete, partial and mild AIS (CAIS, PAIS and MAIS) according to the severity [30, 55].

Spinal and bulbar muscular atrophy

X-linked spinal bulbar muscular atrophy (SBMA) or Kennedy’s disease, is a disorder of the motor neurons characterized by the adult onset (usually 30-50 years of age) and the slow progression of proximal muscle weakness and atrophy associated with tremor, muscle cramps, and weakness, mainly of the tongue, facial muscles, and proximal limb
girdle muscles [12, 63-65]. Moreover, affected males frequently present with gynecomastia, reduced or absent fertility with oligozoospermia or azoospermia, and high plasma levels of LH, FSH and estradiol [64-66]. Such clinical and biological profiles are compatible with an AR defect, as observed in PAIS. Affected individuals have normal fetal masculinization as evidenced by normal male external genitalia and are often fertile in early adulthood [66]. The characteristics of mild androgen insensitivity appearing later in life may be related to reduced AR expression and a lower testosterone level observed in elderly men [67, 68]. Notably, signs of androgen resistance are often the earliest manifestation of Kennedy’s disease, preceding the neurological changes [66]. In heterozygous women mild symptoms can be observed, indicating that female carriers are protected by chromosome X-inactivation and/or by a lower androgen level [66, 69, 70].

La Spada et al. demonstrated that the molecular cause of Kennedy’s disease was an abnormal expansion of the CAG stretch in the amino terminal domain of the AR [45]. In all 35 patients studied, the number of glutamine residues was expanded to 40-52 repeats and no expansion was present in the AR genes of 263 controls. The same study demonstrated a correlation between the size of the expanded segment and the severity of the disease, but this correlation only seemed to apply to the neurological features of the disease and not to the features of androgen insensitivity. Important is, that although the morbid gene and the causative mutation leading to Kennedy’s disease is known, the pathogenesis is still unknown [71, 72]. Although patients with Kennedy’s disease frequently present signs of PAIS, SBMA does not occur in subjects with AIS. This is surprising, because androgens are known to play a role in growth, differentiation and regeneration of motor neurons. A possible explanation of these conflicting data is, that the expansion of the polyglutamine region of the AR would result in a gain of a function due to misfolded protein that is selectively toxic for motor neurons, and in a loss of normal protein function required for full androgen response [65].

Male infertility
Approximately 10-15% of all couples have difficulties of conceiving, making infertility one of the most common disorders in the Western world [73]. In 30-50% of the cases, the cause is male related, mainly linked to azoospermia and oligospermia [74]. In a small
proportion of the infertile men, the explanation is to be found within genetic and other molecular abnormalities [74]. Chromosomal abnormalities like Klinefelter’s syndrome (47,XXY) and mixed gonadal dysgenesis syndrome may in a few cases be the cause of the problem. Mutations in the \(AR\) are also sometimes, although not frequently, the cause of male infertility [75].

In some studies also the \(CAG\) repeat length was associated with infertility. Tut \(et\ al\.)., Mifsud \(et\ al\.) and Harkonen \(et\ al\.) reported association between long \(CAG\) stretches, although within normal length, and male infertility [49, 76, 77]. However, others have not been able to find such associations [78-80]. In a meta-analysis from 2007, infertile men were shown to have 0.19 repeat longer \(CAG\) stretches than fertile men [80].

Prostate cancer

In Europe, prostate cancer is the most common solid neoplasm in men, with an incidence rate of 214 cases per 1000 men, outnumbering colon and lung cancer (http://www.uroweb.org) [81]. The true incidence is however difficult to estimate since the malignancy is frequently subclinical, detected only via prostate specific antigen (PSA) testing. Before PSA-based testing was introduced, mortality from prostate cancer was steadily increasing, most likely caused by increasing longevity [82, 83]. However, in the USA, age-standardized prostate cancer specific mortality rates have during the last years decreased to a level lower than before the era of PSA screening [83]. This is also noted in Norway, Sweden and Finland, although rates remain higher than before the PSA test became widespread. On the other hand, in some countries, such as Denmark, mortality from prostate cancer has continued to increase.

There are significant ethnic variations in incidence throughout the world, where African Americans have the highest incidence rates and Asians the lowest rates [84]. Even within countries, there are clear differences in prostate cancer incidence and mortality. In e.g. the USA, the risk-adjusted incidence rate in the 60-69 age group is estimated to be >700 per 100 000 whites and >1200 per 100 000 for blacks [85]. The mortality is also substantially higher in African- than Caucasian Americans [86]. However, although prostate cancer incidence is also higher for UK blacks than whites, there is no evidence that their disease-specific mortality is higher.
Because of the pivotal role of androgens and the AR in development of the normal prostate and in the pathogenesis of prostate cancer, androgen ablation is a cornerstone in the treatment of this disease and numerous investigations regarding AR mutations and rearrangements as well as the CAG and GGN polymorphisms have been performed. The results show, that germline mutations in the AR gene are rare, only two documented to date (http://androgendb.mcgill.ca). With respect to the polymorphic CAG and GGN stretches, the results are conflicting, partly because of populations of mixed ethnicities, low numbers of participants and determination methods of repeat lengths. Some authors report positive associations between shorter CAG repeat length and higher risk for prostate cancer [87]. On the contrary, Bratt et al. and Freedman et al. found no association between CAG repeat length and prostate cancer [88, 89]. The largest study to date on prostate cancer risk and CAG repeat length has been carried out by Lindström et al. and included more than 6000 cases and controls [90]. No association between CAG length and prostate cancer was reported, but a relation between CAG length and circulating levels of testosterone and estradiol was observed. Regarding GGN, several studies indicate that men with short GGN repeats have a higher risk of developing prostate cancer [91-93].

Testicular cancer
Testicular cancer accounts for about 1 percent of all cancers in men from Western countries [94]. Despite its overall low incidence, it is the most common solid malignancy in men between the ages of 15 and 35 years. As in prostate cancer, there are large ethnical differences in both incidence rates and mortality rates, with Caucasians having a significantly higher risk of developing testicular cancer compared with black African and African American populations [95]. Cross-border comparisons of the Organisation for Economic Co-operation and Development countries, showed that the highest testicular cancer mortality rates can be found in Denmark, Norway and New Zealand, and the lowest in Korea and Japan [96]. There are few risk factors known for testis cancer. Although cryptorchidism, infertility and testicular dysgenesis have been proposed as contributing factors, the causes remain largely unknown [97, 98]. A genetic basis, with main focus on the AR gene and the polymorphic CAG and GGN repeats, has been studied
as a possible cause of testicular cancer. However, also regarding this condition the results are conflicting [99, 100].

Aging men
It is generally accepted that testosterone concentration falls with increasing age [101]. Multi-factorial mechanisms have been proposed for this age-related decline in the circulating levels of testosterone. Wu et al. reported an increase in serum LH with increasing age, suggesting testicular failure as an important factor in this process [102]. This idea is also supported by the age-related decrease of the Leydig cells mass [103]. Various other changes in the signaling of the HPT axis are also seen as possible contributors to the fall in testosterone, including disruption of the pulsatile GnRH secretion due to aging, leading to a diminished secretion of LH from the pituitary [104]. Impaired testicular function may also be related to reduced blood supply to the testes with rising age [103]. These explanations are not mutually exclusive and a wide array of other factors such as lifestyle factors and especially obesity has been shown to contribute to the variations of endogenous testosterone levels [101, 105].

Significant association between androgen receptor CAG repeat length and the level of free testosterone has been described in men in various age groups [106-109], although not in all studies [110].

The clinical importance regarding the declining testosterone level among elderly men is widely debated, [102, 111, 112], and there is no widespread consensus as to what types and degree of symptoms that would favor a diagnosis of acquired androgen deficiency in aging men [113]. The 2006 clinical practice guidelines by The Endocrine Society; "Testosterone therapy in adult men with androgen deficiency syndromes" suggest that the so-called late-onset hypogonadism should be regarded as a clinical and biochemical state with rising age, characterized by particular symptoms such as loss of libido and erectile dysfunction, together with a low level of serum testosterone [114]. The authors advise not to treat androgen deficiency in general, but do not advise against the treatment of older men with low testosterone and clear symptoms of hypogonadism [114]. The guidelines and the evidential basis for the diagnosis and treatment of low testosterone in elderly men have, however, been debated and a more clear differentiation between age-
related hypotestosteronemia and late-onset hypogonadism is warranted [102, 111, 112]. The clinical practice guidelines was recently updated [115], favoring a more restrictive approach to less clearly defined hypoandrogenic states. However, the guidelines are still short of aspects concerning possible side effects of androgen treatment and the clinical efficacy remains unclear [116, 117].

Androgens in metabolic diseases

Central obesity, glucose intolerance and high blood pressure are increasingly prevalent throughout the industrialized world, and of major concern as public health issues due to their contribution to the accelerated development of cardiovascular disease (CVD) [118]. These risk factors for both type-2 diabetes and CVD tend to occur together, and define the metabolic syndrome [119, 120]. Guidelines from the 2001 National Cholesterol Education Program-Adult Treatment Panel III (NCEP) [7] defines metabolic syndrome as a clustering of metabolic derangements, including patients with three or more of the following components:

- Abdominal obesity (waist circumference >102 cm)
- Triglycerides >150 mg/dL (>1.7 mmol/L)
- High-density lipoprotein cholesterol (HDL) <40 mg/dL (<1.04 mmol/L)
- Fasting glucose > 110 mg/dL (>6.1 mmol/L)
- Blood pressure >130/85 mmHg.

The prevalence of the metabolic syndrome is rapidly increasing, affecting approximately 30-34% of adults aged 40-75 years in the UK and 35% of all citizens of the United States [121, 122]. The large prevalence of the metabolic syndrome and its association with a number of lifestyle-related diseases suggest a complex, multi-factorial pathogenesis. Given the central roles played by male sex, body fat distribution and fat- and glucose metabolism as risk factors, it is tempting to speculate that androgen signaling might be of relevance to the development of the metabolic syndrome. Change in body composition is associated with lower testosterone [101]. Obesity and especially redistribution of fat mass from peripheral to central intra-abdominal deposits is associated with development of
type-2 diabetes and CVD [123]. Central obesity is a key element of the metabolic syndrome, and is often regarded as a cause rather than a consequence of metabolic dysfunction. Insulin resistance is associated with hyperinsulinemia and hyperglycemia and may further cause development of dyslipidemia and hypertension. However, it is still unclear whether the decline of testosterone in elderly men is a predictor for development of the metabolic syndrome [124], or if the sub-clinical testosterone levels found in men with the metabolic syndrome is an indication of overweight and poorer health. For instance, Kauka et al. [125] and Niskanen et al. [105] have shown an increase in free testosterone levels among obese men with the metabolic syndrome undergoing a rapid and significant weight loss.

Independent associations between the AR gene polymorphisms and the components of metabolic syndrome have been found, such as shorter CAG repeats and low HDL levels [126, 127] suggestive of an unfavorable lipid profile. However, despite this potentially higher risk profile for atherosclerosis, Hersberger et al. found no evidence of increased risk of coronary heart disease in carriers of short CAG repeat sequences [126]. Thus, apart from its relevance in SBMA, the importance of the CAG repeat length was at the start of this project unclear in a clinical perspective.
Aims of the thesis

The overall aim of this work was to examine the relationship between AR CAG length and androgenic effects, both in vivo and in vitro. Specific aims were:

• To examine whether CAG/GGN repeat lengths have an impact on endogenous testosterone and LH levels in elderly men participating in the Tromsø study.

• To investigate to which degree the AR CAG and GGN repeat length polymorphisms may be associated with the metabolic syndrome as well as with the individual components of the metabolic syndrome in elderly men.

• To examine the AR CAG lengths’ influence, if any, on AR activity by comparing three AR genotypes with specified CAG lengths in vitro.

• To investigate whether serum concentration of PSA and PSA expression in the prostate gland is associated with CAG length.
Methods

Ethical considerations

All participants were provided with a written informed consent agreement prior to the examinations. The studies were approved by the Tromsø Regional Research Ethics Committee and the ethical review board of Lund University, respectively.

Study populations

Elderly men (Paper I, II and IV)

The Tromsø Study is an ongoing populations based health survey of the inhabitants from the municipally of Tromsø, mainly focusing on lifestyle related diseases. The study was initiated in 1974 as a response to the high CVD mortality in Norway (Tromsø 1). Since then, the study has been repeated five times, in 1979-80 (Tromsø 2), in 1986-87 (Tromsø 3), in 1994-95 (Tromsø 4), in 2001-02 (Tromsø 5) and in 2007-08 (Tromsø 6) [128].

In 2005, all men aged 60-80 years who participated in the fifth survey and had a serum total testosterone level ≤11 nmol/L (no. 335) were invited to participate in the clinical study, “Older men and testosterone”. For each subject, a randomly assigned age-matched control with normal levels of testosterone was also invited. The invitation letter informed about the purpose of the study, but did not reveal the subject’s testosterone level. Of the 157 men who attended this subsequent follow-up examination, 69 still had testosterone levels ≤11.0 nmol/L, and were included in the study. 124 men with normal testosterone levels in 2001 also attended the subsequent follow-up and 104 individuals who still had normal levels of testosterone were included as the control-group.

As testosterone levels have been shown to decrease with increasing age [101] it was unexpected that more then half of the men with low testosterone levels in 2001 were found to have normal levels four years later. This may be due to the phenomenon regression towards the mean; meaning that variables may vary more at their first measurement, but tend to close up to the mean value at a second measurement [129, 130]. Another plausible explanation may be related to the diurnal variation of serum testosterone. The blood samples from 2001 were drawn at any time point between 0800 and 1800 h, while preferably all samples should have been drawn in the morning when
testosterone levels are at their highest. A recent study by Brambilla et al. reported a reduction of 10-25% in serum testosterone from 0800 to 1600h, depending on the subjects age [131]. Thus, if testosterone was measured in the afternoon in 2001, the participant could have been interpreted to have a low testosterone level, even though a sample drawn in the morning in the same individual might have been normal.

Military conscripts (Paper IV)
Previously, approximately 95% of all 18 year old Swedish men had to undergo a medical health examination prior to military service. As only those with serious chronic diseases were excluded a priori, this group of conscripts closely reflected the general population of young Swedish males. A total of 2255 men born between 1979 and 1982 and living within 60 km of the city of Malmö in Southern Sweden were asked to participate in a study on reproductive health, and 305 men (13.5%) agreed to enter the study [132]. The subjects included in the study were all aged between 18 and 21 years (mean 18.2).
A blood sample was drawn in the morning for both hormonal measurements and DNA extraction. Circulating levels of FSH, LH, SHBG, testosterone, PSA and estradiol (E$_2$) were measured in all participants on an automated fluorescence detection system (Autodelfia, Wallac Oy; Turku, Finland) at the routine clinical chemistry laboratory, Uppsala University Hospital. Intra- and total-assay CV values were below 4 and 7.5% respectively. The study protocol also included a semen sample.
As the AR gene is located on the X chromosome, in order to exclude any impact of ethnic variation, genotyping of the CAG and GGN repeats was performed in all men with mothers of Swedish origin (n = 223). Of these, serum PSA was available for 187, who consequently were included in current study.

Men screened for prostate cancer (Paper IV)
Prostate biopsy material was obtained from a selection of 134 men selected from a PSA screening program at the Urological Clinic, Skåne University Hospital, Malmö, Sweden to undergo further diagnostic evaluation based on supra-normal (>4 ng/mL) levels of PSA. Measurement of PSA in serum of the men in the screening program was performed as in the military conscripts. The men were born between 1910 and 1952, with a median
age of 67 years. Eighty-nine patients showed evidence of prostate cancer, whereas 45 had histological changes consistent with benign hyperplasia of the prostate [133]. All biopsies were evaluated by a pathologist. All stages and grades of prostate cancer were included. Follow-up was median 29 months for patients with BPH and 41 months for cancer patients. Nineteen randomly selected biopsy samples were used for this study. Nine men had $CAG_{14-18}$, five had $CAG_{22}$, and five had $CAG_{26-28}$. The biopsies were taken for prostate cancer diagnosis purposes before any eventual treatment was started. The samples were collected during the period 1997-2002 and had been paraffin embedded. Two of these samples only contained benign tissue and two samples only had malignant tissue.

Blood sample collection and analyses (Paper I, II and IV)
Serum samples were consecutively analyzed according to standard protocols at the University Hospital of North Norway, except from the EDTA buffered blood samples, which were stored at -20°C awaiting further DNA analysis. Serum concentrations of estradiol, FSH, LH, PSA and total testosterone was determined utilizing an automated electrochemical luminescence immunoassay apparatus (Modular E, Roche Diagnostics GmbH, Germany). SHBG, insulin and C-peptide were measured by an automated chemoluminescence-based assay (Immulite 2000, Diagnostic Product Group, USA). Free (unbound) testosterone was calculated using the obtained values for total testosterone and SHBG using a fixed albumin value according to the formula of Vermeulen et al. [134]. Glucose, serum cholesterol, low-density lipoprotein (LDL), HDL and triglycerides were assayed using an automated clinical chemistry analyzer (Modular P, Roche Diagnostics GmbH, Germany). Glycosylated hemoglobin (HbA1c) was measured using a high performance liquid chromatography method (Variant II, Bio-Rad Labs, USA).

Genetic analyses (Paper I-IV)
DNA extraction
In this study, genomic DNA was prepared from peripheral leukocytes by a rapid purification procedure as follows: 50 µl frozen blood was thawed, mixed with 400 µl
freshly prepared 0.17 M NH₄Cl, inverted and left in room temperature for 20 min. The samples were spun for 30 sec and the pellet washed 3 times in cold 0.9% NaCl. Finally the pellet was suspended in 200 µl 0.05 M NaOH, boiled for 10 min and neutralized with 25 µl 1 M Tris-HCl (pH 8.0). Five µl was used for each polymerase chain reaction (PCR).

CAG and GGN nucleotide repeat analysis
In the present thesis, assays of AR CAG and GGN polymorphisms were performed according to the protocols of Lundin et al. [43]. Briefly, the GGN repeat was amplified for 35 cycles in an Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany), under the following conditions: denaturation at 96°C for 45 s, 45 s annealing at 61°C and 1 min extension at 72°C, with an initial denaturation step at 96°C for 3 min and a final extension step at 72°C for 5 min. The sequence of the forward primer was 5´-CGGTTCTGGGTCAACCTCA-3´ and the reverse primer sequence was 5´-TCACCATGCGCCAGGGTA-3´.

The CAG repeat was also amplified in a PCR with the forward primer: 5´-TTAGGGCTGGGAAGGGTCTA-3´ and the reverse: 5´-TGGGGCCTCTACGATGGGCT-3´. The Samples were amplified using the following program: initial denaturation at 96°C for 3 min; 35 cycles of 1 min denaturation at 96°C, annealing at 61°C for 45 s and extension at 72°C for 5 min.

PCR products were purified, directly sequenced with the reverse primer from the PCR, precipitated, re-suspended and run on an eight-capillary Applied Biosystems (Applied Biosystems, Stockholm Sweden) sequencing gear.

Plasmid construction (Paper III)
In this thesis PCR was used for amplification of the human AR 23 GGN region, which was selected to generate an expression plasmid with the combination 16 or 28CAG and 23GGN. A plasmid expressing an androgen receptor with 22CAG and 23GGN repeats was already available in the research laboratory. These genotypes were chosen to compare the median CAG length in combination with the most common GGN length in
white men \(22\text{CAG}\) and \(23\text{GGN}\) to longer and shorter \(CAG\) repeats within the normal range.

The DNA template for the PCR was human DNA and the reaction was run under the following conditions: 1 min denaturation at 96 °C followed by a 1 min annealing at 56 °C and 3 min extension at 72 °C. The sequence of the forward primer was \(5\text{'}-\text{CCAGAGTCGCGACTACTACAACCTTTCC-3\text{'}}\) and the reverse primer sequence was \(5\text{'}-\text{CCAGAACACAGTGACTCTGCCC-3\text{'}}\).

The PCR products were digested with Kpn1 and BstE11/Eco911, resulting in a 241 bp product, only containing the required \(GGN\) stretch.

Two pCMV4 expression vectors containing full length \(AR\) cDNA with the required \(CAG\) repeat length in combination with an unwanted \(GGN\) repeat were digested with the same enzymes as the PCR product to remove the \(GGN\) repeat present. The opened plasmids, lacking the \(GGN\) repeat, were then purified.

The digested and purified PCR product \((23\text{GGN})\) was ligated into the opened pCMV4 expression vectors, resulting in two vectors with \(23\text{GGN}\) in combination with 16 or 28 \(CAG\) repeats. These plasmids were transfected into \textit{Escherichia. coli} DH5α by electroporation, amplified and purified. Finally, correct incorporation of the \(GGN\) fragment and the \(CAG\) sequence was verified by direct sequencing on an ABI Prism 3730 genetic analyzer (Applied Biosystems, USA).

\textbf{AR transactivation assay (Paper III)}

In the current work african green monkey kidney cells (COS-1) were used for transient transfection of \(AR\) expressing plasmids. COS-1 cells were chosen as they do not express an endogenous \(AR\), are easy to transfect, and have been used in similar studies previously [47, 49]. They express the SV40 large tumour antigen that enables transcription initiation at the SV40 origin site in the pCMV4 expression vector [135]. This results in a high copy number of the vector with the \(AR\) cDNA in each transfected cell [136].

The methods used for transfection of plasmids into mammalian cells are based on various ways of promoting DNA uptake. In methods using calcium phosphate, small precipitates are formed between the DNA and calcium that then are adsorbed by the cell through a
poorly understood mechanism [137]. Electroporation, when cells are given a short electric shock, is an efficient method to transfer DNA directly into the cell nucleus [138]. In liposome based methods, a complex is formed between the DNA and lipids. The complex formed fuses with the cell membrane or is taken up into the cell by endocytosis [139]. We used a transfection reagent containing a blend of lipids (Fugene 6, Roche Diagnostics) and tried various combinations of plasmid concentrations in order to optimise the conditions.

For transfection 150 000 cells were seeded in 12-well plates. The cells were grown for 24h and subsequently co-transfected with 600 ng pCMV4 plasmid containing the AR genotype of interest, 200 ng of the reporter plasmid pGL3hPSALuc2 and either 1 ng of pCH110β-gal or 5 ng pRL-SV40 renilla as transfection efficiency controls. The pCMV4 plasmid has a strong cytomegalovirus promoter that, as mentioned earlier, can be replicated in COS-1 cells. The reporter plasmid had a human PSA-promoter in front of the luciferase gene. Since renilla, β-gal and various other plasmids often are used as transfection efficiency controls, our intention was to investigate if the results could be influenced by any common transfection efficiency plasmid.

Cells were grown and kept in Dulbecco’s modified eagle media containing 10% heat inactivated fetal bovine serum supplemented with 2 mM glutamine and 0.02% gentamicin.

After transfection, cells were left for 24 h before they were washed and new media with 10 or 100 nM of DHT or R1881, or media with no hormone was added. Since the COS-1 cells are known to metabolise testosterone, Methyltrienolone (R1881), which is synthetic testosterone that can not be converted into DHT by 5α-reductase, was used to study the effect of testosterone. The serum used in the media was stripped from hormones.

Luciferase and renilla activities were assessed by the Dual Luciferase Reporter assay, whereas the combination of β-gal and luciferase was assessed by the Dual Light Luciferase assay. Total protein amount in each sample was measured by the method of Bradford [140], which is a rapid and sensitive method for the quantitation of microgram
quantities of protein utilizing the principle of protein-dye binding. The experiment was repeated up to 31 times with DHT and six times with R1881.

To rule out the possibility that differences in activity depended on varying AR transcription amount, mRNA was prepared from transfected cells. The mRNA was then reverse transcribed to complementary DNA (cDNA) and quantified by qPCR.

Quantitative real-time PCR analysis (Paper III)

For the measurement of AR expression in COS-1 cells, qPCR using a fluorescent dye was performed. Total RNA was extracted from transfected COS-1 cells using the commercially available RNeasy mini kit (Qiagen, Sweden). During the process of RNA extraction, the RNA was treated with DNase to ensure that no residual plasmid DNA remained, which could be used as false template in the subsequent qPCR reaction. Primers that specifically bind to the polyA tail of mRNA molecules were used in the reverse transcriptase reaction to synthetise cDNA with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Sweden), utilizing an oligo-dT primer. The AR specific primers in the qPCR reaction were designed to be intron/exon spanning to facilitate the discrimination of products from chromosomal DNA and cDNA. Primers used for AR detection were, forward 5´-AGCCTATTGCGAGAGAGCTG-3´ and reverse 5´-GCTTCACTGGGTGGAAAT-3´.

The chosen endogenous gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was detected with the forward primer 5´-CGACCACTTTGCAAGCTCA-3´ and reverse primer 5´-AGGGGTCTACATGGCAACTG-3´. The AR mRNA was quantified by using the comparative $C_T$ method. The method is based on normalization of fluorescence values for the gene of interest to the values for the endogenous control. Three samples of each genotype were analysed in triplicates and the data were compared with the expression of 22CAG treated with 10 nM DHT, which was set as a reference value and included in each qPCR run.

Enzyme linked immunosorbent sandwich assay (Paper III and IV)

Methods for estimating or determining specific protein amount most often are antibody-based techniques. Western blot is commonly used for detection of proteins in cell lysates.
It is a combination of fractionating the proteins in the sample based on molecular weight by polyacrylamide gel electrophoresis, in which all separated proteins are transferred to a solid membrane and specifically detected by using primary antibodies and secondary antibodies conjugated to a detectable reagent. The technique is valuable for detecting specific proteins, but is a crude method for determining the specific protein amount. Instead, enzyme-linked immunosorbent sandwich assay (ELISA) can be used. The sandwich ELISA allows accurate determination of protein amount. It is an antibody-based method where specific antibodies are used to coat a microtiter plate. The sample is added, the protein of interest (antigen) is bound to the antibody and the unbound residual material in the sample washed away. The amount of bound protein is determined by using a second antibody conjugated to a colorimetric, enzyme linked or fluorescent/chemiluminescent antibody, which develops a measurable colour/fluorescence when substrate is added. To increase the sensitivity of the assay, an unconjugated secondary antibody can be used before a third conjugated antibody is added. Standard samples, with known antigen concentration, can be included in the reaction to give a standard curve. This curve can then be used to determine the amount of antigen in each sample [141, 142].

In this work a sandwich ELISA was the chosen method to rule out if differences in measured luciferase amount were depending on varying AR protein amount. Since no purified AR protein that could be set as reference is available on the market, the exact amount of AR could not be detected. We therefore used relative amounts with 22CAG as reference. The appropriate amount of total protein to be used was decided by running a dilution curve on one sample before the analysis of all samples. The dilution curve showed which dilution to use to obtain optical density values within the linear range.

In all the ELISA reactions, lysates from the prostate cancer cell line LnCaP were included as positive controls. Untransfected COS-1 cells were used as negative controls.

**Time-resolved fluorescence imaging (TRIF, PaperIV)**

Serum PSA measurement is commonly used to screen for prostate disease and in the follow up of patients with prostate cancer. Expression of the *PSA* gene is regulated by the AR. In order to determine if AR expression and activity is varying depending on *CAG*
length, the objective was to measure AR and PSA protein amount directly in human prostate tissue from men with known genotype. For this purpose, common immunohistochemistry can be used, but the signal obtained is not always linear to protein amount and the background is often high [143].

Instead, the chosen method for direct quantification was TRFI. This is a method where specific antibodies directly or indirectly linked to lanthanides are used in combination with image acquisition in an epifluorescence microscope. The lanthanides can be excited at specific wavelengths and have a long decay time. The long decay time makes it possible to obtain images with low background noise and autofluorescence [143]. In comparison to immunohistochemistry, TRFI gives a linear relation between signal intensity and the specific protein expression; it also allows an automated and improved quantification and evaluation of cellular parameters [143].

First the method was verified on AR transfected and paraffin embedded COS-1 cells treated with 10 nM DHT. The cells were transfected as mentioned earlier with ARs harbouring 16, 22 or 28CAG in combination with 23GGN. After harvesting and fixation the cells were paraffin embedded. Sections were then prepared for AR protein measurement. The primary antibodies were directed against AR and PSA. They were conjugated to Europium (Eu) and Terbium (Tb), respectively, making it possible to measure both AR and PSA amount from the same sample.

For the prostate biopsies, TRIF was performed as described above, followed by hematoxylin/eosine staining and standard histological analyses. One sample from each patient was analysed once. The primary areas of interest were prostate glands, where AR expressed in the nucleus of epithelial cells and PSA found in the stromal cells could be detected. The Eu signal was obtained with an emission filter set at 615 nm and for Tb, a filter at 545 nm was used. This gave separate images with specific Eu and Tb signals from the same area. These images were then analysed, resulting in specific density values for each region of interest in every image. The mean intensity of the background was subtracted from each region of interest making it possible to compare the images to each other.
Statistical analysis

All statistical tests were two-tailed with statistical significance defined as $p < 0.05$. The data were analysed using the SPSS statistical package for Windows version 15.0 (SPSS Inc., Chicago, IL, USA).

In studies I-II, normal distribution was evaluated with determination of skewness and histograms. Total and free testosterone and SHBG were considered to be normally distributed. LH was slightly skewed, but because it was not used as a dependent variable it was not log-transformed. In study I, men with subnormal and normal testosterone levels were compared using $t$-test. Age-adjusted and BMI-adjusted partial correlations were used when analyzing for univariate associations. Analysis of variance was used to calculate means of total and free testosterone and SHBG by quartiles of $CAG$ and three groups ($< 23; 23; > 23$) of $GGN$ repeat lengths.

In study II, men with subnormal and normal levels and men with $CAG$ repeat lengths $\leq 21$ and $> 21$ and three groups of $GGN$ repeat lengths ($< 23; 23; > 23$) were compared using Student's $t$-test. Pearson correlations were used when analysing for univariate associations. Multiple regression analyses were performed to identify independent predictors of metabolic syndrome components. ANOVA was used to calculate mean adjusted $CAG$ repeat lengths according to components of metabolic syndrome.

In study III, the activity of the most common genotype 22$CAG$ stimulated with 10 nM of ligand was set to 100% and the mean activities of other genotypes were expressed relative to this. The results are presented as means (ranges). 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 (22$CAG$) and the other genotypes.

In order to make the in vivo results comparable to our in vitro findings, serum PSA levels in the 22$CAG$ group, corresponding to the median length in Caucasians, were used as reference in study IV. The two groups – adolescents and elderly men – were analysed separately. The results are presented as means (SD). Univariate regression test was used
to test for differences in PSA levels between those having \( CAG<22 \) or \( CAG>22 \) and the reference group. The association between \( CAG \) number and PSA was also tested with the repeat length as continuous variable in partial correlation analyzes. \( GGN \) number and testosterone level was included in the analysis as confounder. The tissue samples were also grouped according to their \( CAG \) length, representing short \( CAG \) stretches (\( CAG<22 \)), median length (\( CAG22 \)) and long stretches (\( CAG>22 \)). The mean and median value of all values from one sample and the 25\(^{th}\) percentile and 75\(^{th}\) percentile values were used for statistical calculations.
Main results


In this nested case-control study on elderly men, we wanted to examine whether $CAG$ and/or $GGN$ repeats were significant modulators of serum concentrations of total and free testosterone as well as of LH. $CAG$ and $GGN$ repeat length polymorphism within the $AR$ was mapped within a population of elderly men encompassing individuals with normal (n=104) or subnormal (n=69) levels of serum testosterone. Although there was no significant differences in average $CAG$ and/or $GGN$ repeat length between the two groups, cross-sectional analyses of the total study population showed positive correlation between $CAG$ repeat length and total and free testosterone levels. When adjusting for body mass index or waist circumference the associations were no longer significant. We found no association between $GGN$ repeat length and sex hormones.

**Paper II.** Androgen receptor gene polymorphism and the metabolic syndrome in 60-80 years old Norwegian men.

In this study we wanted to investigate to which degree the AR $CAG$ and $GGN$ repeat polymorphisms might be related to the metabolic syndrome. In cross-sectional analyses, men with $CAG$ repeat lengths ≤21 had significantly higher fasting glucose, C-peptide and glycosylated hemoglobin (HbA1c) levels (all $p < 0.05$). In multiple regression analyses, $CAG$ repeat length was an inverse and independent predictor of glucose after an OGTT and of HbA1c levels. We also found that men with more than one component of the metabolic syndrome had shorter $CAG$ repeat number ($p$ for trend 0.013) than those with only one component. Men with metabolic syndrome (≥ three components of the NCEP) had lower $CAG$ repeat number, $p = 0.001$. There were no associations with $GGN$ repeat length.
**Paper III.** CAG repeat number is not inversely associated with androgen receptor activity in vitro.

Our objective was to test the generally accepted hypothesis of a negative linear association between androgen receptor function and the CAG repeat numbers. We studied ARs with CAG lengths within normal range (16, 22 and 28) in a reporter-assay with the human prostate specific antigen promoter as target. With β-galactosidase as transfection control, 22CAG had the highest activity (set to 100%) compared with 16CAG [mean 78% (range 41–132), p = 0.005] and 28CAG [68% (26–162), p = 0.006]. In these experiments the empty control vector also showed high activity (86%, 10-166). When adjusting for renilla-luciferase, 16CAG behaved similar to 22CAG [104% (56–165), p=0.7] and 28CAG having lower activity [59% (33–101), p=0.004]. When adjusting for AR protein, the 22CAG genotype generated the highest activity; 16CAG and 28CAG displaying 20% (10–47, p<0.0001) and 12% (5–21, p<0.0001) thereof. Similar results were obtained with adjustment for total protein. By normalizing for AR-content, contrary to various control vectors, the highest AR activity was confined to the 22CAG and not 16CAG.

**Paper IV.** Androgen receptor CAG length dependent amount of prostate specific antigen in serum and in tissue.

The release of PSA is regulated by androgens acting via the AR. The AR’s effectiveness depends on availability of and on polymorphic CAG repeat in the transactivating part of the AR. Recently, a non-linear relation between the AR’s ability to drive a PSA-reporter gene and the CAG length was reported, suggesting that the AR containing median CAG-length was the most effective compared to AR’s with shorter or longer CAG repeats. Our objective was to investigate whether this in vitro finding also was reflected in PSA concentration in serum as well as in the prostate gland.

In conscripts carrying CAG22, mean serum PSA concentration was 0.86 ng/mL, whereas PSA levels in those with CAG<22 was 0.64 ng/mL (p=0.040) and in men with CAG>22 0.59 ng/mL (p=0.015). Also in older men, PSA was highest in carriers of CAG22, 5.35
ng/mL. Those with shorter repeats had 3.24 ng/mL (p=0.08), whereas carriers of $CAG_{>22}$ had mean serum PSA 2.22 ng/mL (p<0.003).

In non-malignant prostate tissue samples from men with $CAG_{22}$, 47% and 22% less AR protein, respectively, was present compared to short and long repeats, but despite that 15% higher PSA amount was found compared to both shorter and longer repeats ($CAG_{<22}$, p=0.36; $CAG_{>22}$, p=0.81).

Our study shows a $CAG$ length dependent production and blood level of PSA. These findings may have implications for interpreting the results of PSA screening programs.
General discussion

The most important goal of this thesis was to examine the relationship between AR \textit{CAG} number and androgenic effects, both \textit{in vivo} and \textit{in vitro}.

Ever since the finding of vanishing spermatogenesis in men with Kennedy’s disease, in particular the \textit{CAG} segment has been extensively studied with respect to disorders of the male reproductive system. Some \textit{in vitro} studies in the late 1990-ties were interpreted as supporting this hypothesis, which led to the assumption that the \textit{CAG} stretch is inversely associated with AR function, so that the activity was thought to diminish with increasing \textit{CAG} number. In general, however, \textit{in vivo} data based on this hypothesis showed no or limited impact of \textit{CAG} number on phenotypic characteristics and has generated numerous studies on the risk of infertility [78], prostate cancer [80] and serum testosterone levels [144] with conflicting results.

CAG repeat length and androgen status in aging men

The provision of sufficient levels of androgens in aging men is essential to ensure maintenance of male sexual health, demonstrated by the variety of symptoms associated with low levels of circulating testosterone. Hypogonadism has also been associated with lifestyle-related disorders, including diabetes and CVD, particularly in the constellation referred to as the metabolic syndrome, as discussed above.

Within the field of andrology there has been considerable interest in the possibility of treating states of testosterone insufficiency by supplying exogenous androgens. The scientific evaluation of such strategies is hampered by a lack of consensus definition of testosterone insufficiency, especially in aging men. Additionally, our insight into the pathophysiological basis for such age-related testosterone insufficiency remains incomplete. Based on the widespread belief that AR activity is directly correlated to \textit{CAG} repeat length, it has been proposed that the age-related decline in testosterone levels might be exaggerated in individuals harboring short \textit{CAG} repeat sequences, as suggested by some previous studies [107].
Our first objective was to evaluate if polymorphisms in the AR should be taken into consideration when clinically evaluating testosterone and LH concentrations in elderly men. Our data demonstrate a weak positive correlation between CAG repeat length and total and free testosterone levels (Paper I). This is in accordance with the results of a larger, recent study on 2878 men aged 40-79 years, recruited from eight different European countries, the European Male Aging Study (EMAS). The authors also suggested that the increase of circulating testosterone levels in men with longer repeats can adequately compensate partly or totally for the lower AR activity to prevent apparent deficiency of androgen action [144]. Our cohort of men utilized in Paper I was selected from the Tromsø study population based on their testosterone levels, and consisted of men with subnormal testosterone (≤11.0 nmol/L) and men with normal testosterone levels (>11.0 nmol/L). The cutoff value for testosterone to define clinically relevant androgen deficiency is not clearly defined [145]. This is of importance not only in comparing data from different reports, but also for the interpretation of the clinical significance of the data obtained. In addition to evaluating differences between the groups, we also examined the whole cohort in cross-sectional analyses. When studying genetic polymorphisms, adjusting for external factors may be inappropriate, as environmental and lifestyle factors would not be expected to affect the genomic constitution of an individual. However, testosterone levels are for example closely related to measures of adiposity, and we therefore show data both with and without adjustments. Furthermore, growing insight into the issue of epigenetics has led to a less clear-cut distinction between intrinsic and external factors when looking at phenotypic consequences of genetic factors. Our finding that adjusting for BMI or WC eliminates the association between testosterone levels and CAG repeat length points at components of the metabolic syndrome as a possible link between AR characteristics and testosterone levels. The mechanistic basis for this association remains uncertain, but it is well recognized that adipose tissue influences the hormonal state, especially in elderly individuals.

Androgen receptor structure and the metabolic syndrome

Androgen status appears to influence risk of the metabolic syndrome [146], and it was therefore logical to investigate whether GGN- and/or CAG polymorphism could
constitute a risk factor for the development of the constellation of metabolic changes and risk factor complexes referred to as the metabolic syndrome.

Our result showed consistently that men with lower CAG repeat lengths were associated with an unfavorable metabolic profile. Thus, men with CAG repeat length ≤21 had significantly higher fasting glucose, C-peptide and glycosylated hemoglobin (HbA1c) levels, and CAG repeat length was inversely and independently associated with glucose after an OGTT and of HbA1c levels. We also found that men with more than one component of the metabolic syndrome had shorter CAG repeat number than those with only one component, and finally men with metabolic syndrome had lower CAG repeat number. Our results point in the same direction as those reported by Zitzmann et al. and Hersberger et al., who found that shorter CAG repeat length is associated with important factor predisposing to the MS [126, 127]. In these two independent case-control studies, consisting of 110 healthy Caucasian man with mean age 28, and 544 Caucasian men, age spanning from 43-70, HDL-cholesterol levels were found to be positively associated with the number of CAG repeats. In contrast, other studies challenge our findings of an inverse relationship between CAG and the occurrence of the metabolic syndrome or components thereof [144, 147]. In a more resent study Zitzmann et al. [147] demonstrated that a low number of CAG repeats would be beneficial with respect to metabolic parameters. The report showed a positive independent correlation of the CAG repeat number with body fat content, leptin and insulin. Campbell et al. [148] reported a positive association between CAG repeat length and waist circumference. The largest study by far, the EMAS study, found no relationship between CAG repeat length and anthropometric measures, and the CAG/hormone relationships were similar in obese and non-obese men. Nor did they find any evidence for a relationship between CAG repeat length and blood pressure, insulin sensitivity, cardiovascular disease or blood lipid with the exception an inverse correlation with triglycerides. On the other hand, this multicenter report did not investigate the possible relationship between the CAG repeat polymorphisms and the metabolic syndrome per se [144]. The study population was randomly recruited from eight different European countries, rising question of effect of race and ethnicity as a possible reason for inconsistency findings compared to our report. Compared with our cohort the EMAS study also included younger men (40-79 yr.) and mean age was lower (59.9 yr.) as
compared to the participants in our study (69.1 yr.). As the number of men developing components of the metabolic syndrome increase with age, it is possible that the lack of associations in the EMAS study could be related to the younger cohort. Finally, one study reported low CAG repeat numbers to be associated with both beneficial and unfavorable metabolic parameters, thus, suggesting that the influence of CAG polymorphism on overall metabolic status might be complex and non-uniform [149].

CAG repeat length and androgen receptor activity

Based on our present in vitro data, it seems apparent that the assumed inverse linear correlation between CAG repeat length and AR activity does not hold within the normal range of repeat sequence lengths. The assumption of linearity stems largely from experiments in which normal sequence lengths were compared to the extreme trinucleotide expansions seen in patients with Kennedy’s disease. In the study by Chamberlain et al. from 1994, CAG lengths of 25, 35, 49 and 77 were compared when using the viral mouse mammary tumor virus (MMTV) long terminal repeat promoter and stimulating with a supra physiological concentration of DHT [48]. There was a significant difference in transactivation between the shortest and the two longest repeats, which both are in the range of patients with Kennedy’s disease, but no significant difference in activity between those in the upper normal range (25-35CAG). In the study by Tut et al. from 1997, the longest repeat (31CAG) displayed decreased activity compared to the shortest (15CAG), but neither differed in capability to activate the reporter gene compared to constructs of intermediate sequence length (20CAG) [49]. Since the former in vitro studies mostly were based on extreme CAG lengths and reporter-systems containing viral promoters as well as different control vectors, the objective of our third study was to investigate ARs with CAG lengths within normal range (16, 22 and 28) in a reporter-assay with the human PSA promoter as target (Paper III). We also wished to elucidate whether the interpretation of the results was depending on the methods used for adjustment of transfection efficiency and protein content. The choice of the COS-1 cell line for our in vitro studies were based primarily on the fact that they show no endogenous AR expression, as well as technical considerations from preliminary experiments, as further discussed above.
The results showed that the average allele length, CAG22, was associated with superior transcriptional function of the AR compared to shorter (CAG16) and longer (CAG28) variants. The result was parallel to previous findings concerning the other polymorphic region in exon 1 - the GGN repeat. Also for this triplet, the most common variant (23GGN) was in vitro found to give the most efficient receptor [52]. In support of this finding is also the recently published report on reanalysed data on nearly 4000 infertile men, in which a higher risk of infertility was observed in men with CAG lengths in the lowest and highest regions of the normal range [150].

Repeating the experiments in other cell lines, primary cell lines derived from humans, might have provided additional support for our findings, but given the relatively crude nature of the readout assay, it seems likely that results from this immortalized cell line would be applicable to other cell types. Follow-up studies should also evaluate other androgen-responsive promoter elements, which would considerably substantiate the findings of the present work.

In light of the current result on the relationship between CAG repeat length and AR activity, it would be interesting to reanalyze preexisting data by a two-way analysis looking for a shift of CAG repeat sequence distribution away from the 22CAG length that seems to provide optimal AR function.

The regulation of AR activity is determined by the sum of a number of influences, including genetic composition (such as CAG/GGN polymorphism), mRNA transcription and half-life as well as rate of protein synthesis and degradation. Our in vitro data draws attention to a number of methodological considerations when assaying AR activity. Extending these observations to the in vivo setting, it is important to recognize that several levels of regulation, including epigenetic factors such as DNA methylation and RNA interference could be important in controlling the net AR activity at various stages of development, including during aging. These issues have not been addressed in the current work, but call for caution when inferring a mechanistic link between trinucleotide repeat lengths and various alterations in AR signaling.
The question is why both short and long CAG repeats would be of disadvantage? In general, the glutamine length variance is influenced by both specific sequence characteristics and the specific role of the glutamine tract in the protein structure and function, [151]. This is illustrated by the fact that CAG repeats in some genes are polymorphic whereas others are not [152], indicating critical reasons for maintaining a particular CAG repeat length in some proteins. With respect to the AR, there seems to be a length of approximately 10-30 repeats that is endured for proper AR function, and that can be balanced by both AR protein amount, as has been shown previously, [153, 154], and by the secretion of sex hormones in males. In healthy individuals, any impairment in AR functionality can be compensated by activation of the HPT-axis and eventually higher androgen levels.

The AR CAG stretch is in the proteins transactivating domain, which interacts with the hormone-binding domain [40]. The fact that mutations in the CAG stretch can disrupt this interaction [155, 156] or change the protein structure [157] provides evidence that the poly-glutamine tract plays a crucial role in ensuring proper function of the human AR protein. The interplay between the transactivating and the hormone-binding domains has previously been shown to be significantly reduced by shorter or longer CAG repeats than the normal range, generating the hypothesis that these repeats serve as flexible spacers to separate regions of biological activity while maintaining the capacity to interact with co-regulators and the transcription machinery [155]. Such a mechanism could explain how both increased and decreased CAG lengths can influence AR function and why so many conflicting results regarding its physiological impact have been reported.

CAG repeat length and PSA

About 15% of male cancers in developed countries are prostate cancer [158]. The clinical challenges associated with the use of PSA as a marker for prostate cancer are substantial, with a need to improve the process of singling out individuals that will benefit from surgical intervention. In many centers, men with PSA serum levels above 3 or 4 ng/mL are recommended trans-rectal ultrasound and prostate biopsies. Results from two recent randomized trials from Europe and the US, provide strong evidence that PSA-based screening for prostate cancer is associated with a high risk of over-diagnosing. [159,
In the European trial, PSA screening was associated with decreased prostate cancer related mortality but at a great cost; approximately 1,410 men needed to be screened and 48 additional prostate cancer cases would need to be treated to prevent one cancer-related death. Given the high prevalence of subclinical disease and the increasing use of the biomarker as a screening test, the need for refinement of the test’s predictive power is growing. Given the androgen sensitivity of PSA synthesis, it is tempting to speculate that the androgen status of an individual could influence levels of PSA within the tissues and in the circulation.

Our in vitro data suggested that polymorphism in the CAG repeat sequence of the AR influenced signaling in a PSA promoter, with an optimum seen in patients with 22CAG repeats. By comparing this polymorphism and serum PSA levels in a cohort of males, we were able to extend this association to the in vivo situation, demonstrating a corresponding peak in mean PSA levels in individuals with 22CAG sequences compared to both longer and shorter repeats. A similar pattern was found when looking at PSA protein expression in biopsies of patients without evidence of malignant disease. Our data is suggestive of a correlation between a 22CAG repeat length and the presence of higher than average levels of PSA. These findings are adding further challenges to the assumption of an inverse correlation between CAG number and AR activity.

To our knowledge, the CAG22 variant has not been linked to increased risk of any disease of the prostate. Carriers of this genotype, who naturally have higher PSA than counterparts with shorter or longer repeats, would according to current practice be at risk for unnecessary invasive treatment with potential side effects that are associated with a prostate biopsy, including erectile dysfunction and incontinence [161]. On the other hand, carriers of very short or very long repeats could be at risk for not being detected in a PSA-test until incurable state.

Although the association between CAG repeat length and PSA is likely to be mechanistically related to AR activity, it seems unlikely that CAG genotyping would by itself improve the stratification of individuals with supranormal levels of PSA. It is, however, possible that adjusting the PSA cutoff levels based on CAG repeat length would somewhat increase the predictive value of PSA as a marker of malignant disease. Herein lies a possibility of reducing the number of histologically negative biopsies, preventing
unnecessary invasive procedures. The evaluation of such possibilities would require a larger study population, which should be relatively easily achievable given the prevalence of PSA screening procedures and the relative ease and affordability of AR CAG sequencing.

Only few other studies have examined the relationship between serum PSA level and AR CAG length. Mifsud et al. reported a positive association between short CAG repeat lengths and total PSA in subfertile men. Likewise, Giwercman et al. found PSA in seminal plasma to be inversely correlated with CAG numbers, based on 274 healthy military conscripts. These two reports have been conducted on the earlier accepted hypothesis of an inverse correlation between the CAG repeat length and AR activity, making it difficult to compare their results to our study. Further, both reports have a rather mixed study population, of different ethnicities, making generalizations from the results difficult, knowing that the CAG repeat distribution display ethnical differences.

Methodological considerations

In study I and II, the participants were recruited from the general population. The attendance rate in the fifth Tromsø survey was 75.7% in the total male cohort and almost 90% among men between 60-80 years old. Thus, this cohort definitely comprises men from an excellent population-based study. However, the rather low number of men participating in the study must be considered a weakness. We were aiming to include all men with subnormal testosterone and an age-matched control group, but less than half of the invited men attended. In addition, more than half of those who attended did not have persistent testosterone levels when re-examined, leaving us with approximately 20% of the invited cases (30% of the controls). It is likely that a properly conducted meta-analysis of available data would attain sufficient statistical power to provide a clearer picture of the hitherto largely conflicting findings of our results. In this respect, an appreciation of the non-linear relationship between CAG repeat length and AR activity would add another dimension to such a study.

Likewise, the low rate of military conscripts participating in Paper III may reduce the generalisability, although the participants were selected from a general population of
younger men. The low participation rate was probably due to the request for semen samples, very similar to a previous Danish study [162]. However, in the Danish study, blood samples were drawn also from men who did not want to leave a semen sample, and the hormonal values in these men were not different from those who also left a semen sample. We therefore believe that also our cohort reflects the general population in this area of Sweden.

Selection bias can be described as a systematic difference between the two groups which are being studied, or a systematic difference between those included and those excluded from participation in the study [163]. In our case the focus on sexuality and the request for semen sample could possibly have influenced men with a more outgoing attitude to participate, while other would have been more reluctant to participate. The association between higher testosterone levels and risk behavior is well established [164]. Furthermore, in a recent publication, men with shorter CAG repeat lengths had higher self-reported dominance and prestige. Although the association between repeat length and dominance was mediated by physical strength, the relationship between repeat length and prestige was not suggesting that AR polymorphisms may have distinct effects on physical and psychological outcomes [165]. Thus, we can not rule out potential selection bias.

Adjusting for confounding variables is important in statistical analyzes. A confounding variable is an extraneous variable that correlates (positively or negatively) with both the dependent and the independent variable in a statistical model and may therefore influence the validity of the result [163]. However, if adjustments should be made when analyzing the impact of polymorphisms can be questioned. As mentioned above growing insight into the issue of epigenetics has led to a less clear-cut distinction between intrinsic and external factors when looking at phenotypic consequences of genetic factors.

Further, cross-sectional studies are useful for finding associations and creating hypotheses, but do not give answers with regard to causal relationships between exposure and outcome.

Study III was a laborious work, including more than 30 experiments, taking almost one year to finish. The main reason for doing so many experiments was the fact that the topic was controversial and we therefore did not want our results to be questioned due to few
experiments or regarded as by chance findings. The strategy was to first optimise the conditions, repeat the experiments that previously had been done by others [47, 49] and to point out the weak points in previous reports.

For experimental optimisation, only the expression vector containing CAG22 was used, in combinations of 100, 200, 400, or 600 ng expression vector and 600, 400, 200 or 100 ng reporter vector. The amount of DNA that could be transfected was limited by the transfection method (maximum 800 ng) as well as the viability of the cells. Different hormonal stimulation lengths were also tested (24, 48 and 72 h), of which 24h gave the optimal result in terms of AR activity and cell viability.

One of the objectives of the study was also to show alternative, biologically more meaningful ways of performing the experiments by using a physiological promoter and by taking actual AR protein amount into consideration instead of various control vectors. However, although the AR is expressed in many of the tissues in the human body, only a few target genes have been identified. The kallikrein family of genes is one of these, to which PSA belongs, and we had access to the PSA promoter – luc construct. This approach has resulted in a positive response in the research field and a novel strategy of interpreting in vivo data, as was recently discussed in a review article [166].

In study IV, the low number of biopsies is a limiting factor and the reason for lack of statistical significance with respect to AR activity. The reason for this was that very few men were carriers of very short and very long CAG repeats, respectively, in the study group.
**Concluding remarks**

The present knowledge of the relationship between AR *CAG* repeat and androgenic effects is still lacking in many aspects. With the present studies, an additional piece of the puzzle has been added, even though the exact mechanism by which different *CAG* repeat lengths can affect the AR function remains to be fully illuminated.

The main conclusions from the *in vivo* and *in vitro* studies are:

- The *CAG* repeat was positively associated with total and free testosterone, but not with LH. The association was lost after adjusting for BMI or WC.

- The *GGN* repeat was not associated with circulating androgen levels.

- The *CAG* and *GGN* repeats are of minor importance when evaluating androgen levels in elderly men.

- The *CAG* repeat was inversely associated with the number of components of the metabolic syndrome in elderly men.

- Elderly men with the metabolic syndrome had shorter *CAG* repeats lengths.

- The *CAG* repeat lengths affect the trans-activating capacity of the AR, with the average *CAG* length being associated with optimal AR function compared with longer and shorter repeat lengths *in vitro*.

- The serum levels of androgen-sensitive prostate cancer biomarker PSA was higher in men with *CAG22* as compared to those with shorter or longer repeat lengths, in both adolescent and elderly men. Also in non-malignant prostate tissue, PSA was higher in men with average *CAG* length. The results challenge the widespread theory of an inverse correlation between *CAG* number and AR activity, and may also contribute to a better evaluation of the results of PSA screening.
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Paper 3
Paper 4