Genome-wide screening of Biomarkers in Androgen insensitivity syndrome (AIS)

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To my parents

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1. Introduction

1.1 Gender

The birth of a new baby is an exciting event to mankind. The next question arises is it a boy or a girl? The sex can be defined in several ways, such as genotypic sex, phenotypic sex, gonadal sex and sex of rearing. The genetic sex is generally determined by chromosomes, 46,XY or 46,XX. The chromosomes are responsible for the development of male gonads (the testes) or the female gonads (ovaries). The hormones secreted by the gonads are essential for the development of the internal and external genitalia. Thereafter, phenotypic sex is determined solely by the presence or absence of androgen action. Generally 46,XY karyotype is associated with male development and 46,XX with female development. However, rarely sex reversal may take place in case of a male phenotype with the 46,XX karyotype or female phenotype with 46,XY karyotype called 'disorders of sex development' (DSD).

1.2 Embryology of Sex development

1.2.1 Gonadal differentiation

During the early stage of sexual development testis and ovaries are histologically undistinguishable. Therefore, they are called as 'bipotential gonad'. Bipotential gonads arise from the urogenital ridge, which is a derivative of intermediate mesoderm. Testis determination occurs during the 6-7 week of gestation (Figure 1). Testis organizes into two distinct compartments-testicular cords, and interstitial region (Parker K.L *et al*. 1999). Testicular cords are the precursors of the seminiferous tubules. The testicular cords consist of Sertoli cells and primordial germ cells. The Interstitial region consist of Leydig cells and the peritubular myoid cells. In contrast to testis, ovaries exhibit little structural differentiation until late gestation.

Initially, the urogenital tracts are also indistinguishable in male and female embryos. At this stage male and female embryos consist of two sets of paired ducts: The Müllerian ducts and the Wolffian ducts. In males the Y chromosome influences the development of testis. Testis produces specific hormones that trigger the male sexual differentiation (Figure 2). Leydig cells produce androgens and Sertoli cells produce the anti-Müllerian hormone (AMH) (Jost A *et al*. 1953, 1973). Androgens (mainly testosterone and also by weaker

androgen such as androstenedione) influence the Wolffian ducts to differentiate into the seminal vesicles, epididymis and vas deferens (Boehmer ALM *et al*. 1999). AMH regresses the Müllerian ducts. During the 8 week of gestation AMH is secreted and regresses the Müllerian ducts (Baarendo WM *et al*. 1994, Allard S *et al*. 2000). The Müllerian ducts stabilize in the absence of AMH and form the uterus, Fallopian tubes and the upper part of the vagina.

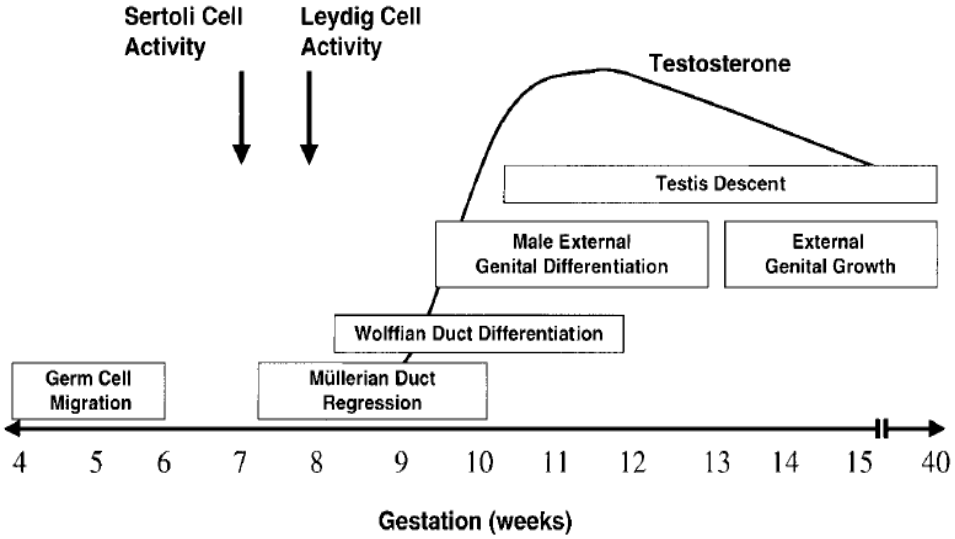

Figure 1. Embryologic events in male sexual differentiation (Hughes A.I, 2001).

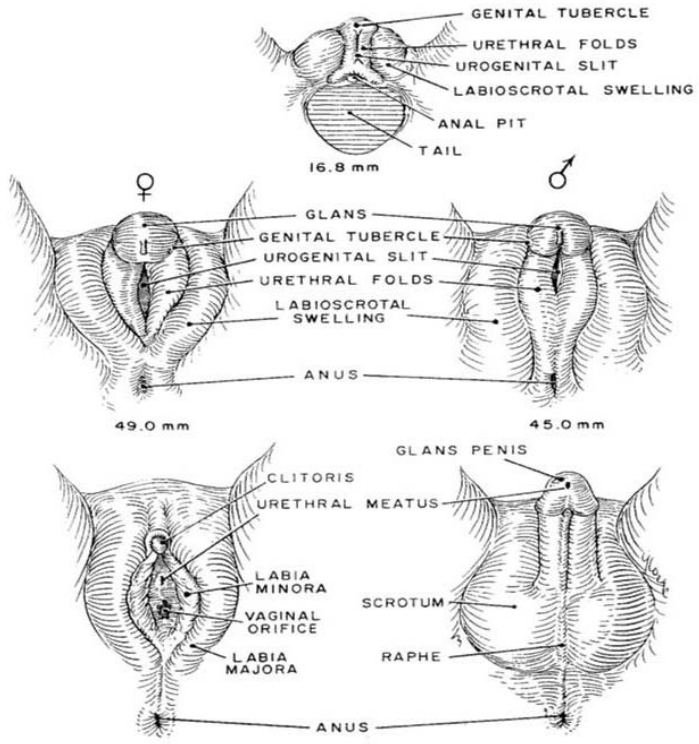

Figure 2. Differentiation of the male and female external genitalia.

1.2.2 Genes essential for gonadal differentiation

The genes involved in gonadal development are partly known. They are sex determining region on the Y chromosome (SRY), SRY box related 9 (SOX 9), DAX 1 (NROB1), Wilms tumor 1 (WT1), LIM1, steroidogenic factor 1 (SF1 or NR5A1), R-Spondin (RSPO1), desert hedgehog gene (DHH), doublesex and mab-3-related transcription factor 1 and 2 (DMRT1, DMRT2) (Figure 3).

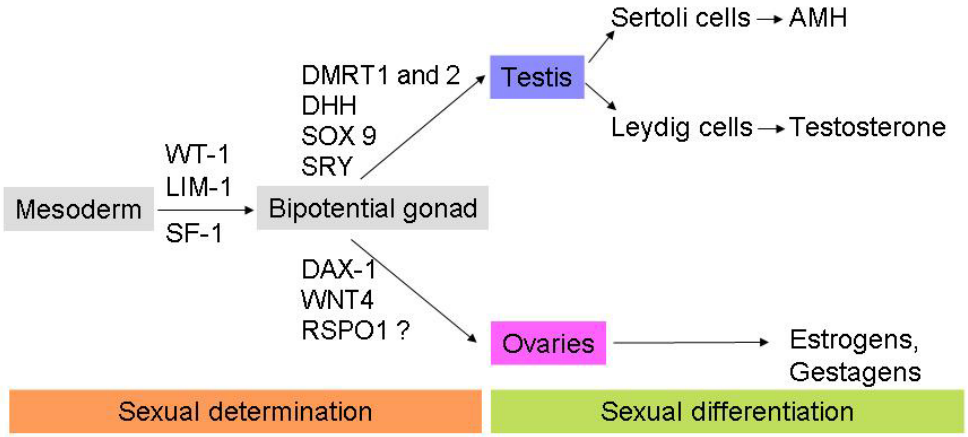

Figure 3. Molecular events in sexual determination and differentiation.

If SRY is expressed in a bipotential gonad it differentiates into a testis (Koopman P *et al*. 1991). A day after SRY expression DAX 1 and WNT4 are downregulated (Harley VR *et al*. 2003). SRY binds to the promoter of anti-mullerian hormone (AMH) gene and controls the expression of steroidgenic enzymes. Probably SRY induces the expression of AMH to prevent the formation of Mullerian duct derivatives. The SRY gene product is an high mobility group box (HMG) protein. It acts as a transcriptional factor and in turn regulates the expression of other genes (Capel B *et al*. 1998).

SOX 9 is the first gene downstream of SRY that displays male specific upregulation and nuclear localization (Harley VR *et al*. 2003). SOX 9 is important for the differentiation and function of Sertoli cells (Vidal VP *et al*. 2001). Defects in SOX 9 lead to sex reversal in 46,XY individuals as well as skeletal malformation known as campomelic dysplasia (Lim HN

et al. 1998). SOX 9 is connected with chrondrogenesis and gonadal differentiation. Regulation of AMH gene requires cooperative function of SF1 and SOX 9.

DAX 1 is an orphan nuclear receptor that lacks an expected DNA binding domain (V. Giguère *et al*. 1999). It acts as a potent corepressor of nuclear receptors (Sablin EP *et al*. 2008) including SF1 (M. Ito *et al*. 1997, P.A. Crawford *et al*. 1998, R. Clipsham *et al*. 2003), Androgen receptor (AR) (Niakan KK *et al*. 2005), Estrogen receptor (ER) (Zhang H *et al*. 2000) and Progesterone receptor (PR) (Agoulnik IU *et al*. 2003). DAX 1 potentially inhibit the ligand dependent transcriptional activation, N and C terminal activation domains of AR (Holter E *et al*. 2002, Agoulnik IU *et al*. 2003). DAX 1 is known to shuttle between cytoplasm and nucleus and capable of relocalizing AR in both compartments (Holter E *et al*. 2002). DAX 1 is repressed by SRY during testicular development (Nachtigal MW *et al*. 1998). It is expressed during ovarian development but is suspended during testicular formation, implying a critical role for this gene in ovarian formation.

WNT4 mutations in humans may lead to partial female to male sex reversal. SOX 9 directly represses the expression of WNT4. WNT4 is involved in the initial steps of Müllerian duct differentiation rather than in the maintenance of the duct (Bernard P *et al*. 2007).

SF1 is involved in endocrine function and development of the adrenal glands and gonadal development in both sexes (Dewing P *et al*. 2002). However, SF1 seems to be particularly important for testis differentiation (Lin L *et al*. 2007). SF1 and SRY cooperatively upregulate SOX 9 (Sekido R *et al*. 2008). Later on, SF1, SOX 9 also binds to the enhancer to help maintain its own expression after that of SRY has ceased. SF1 mutations in children, adults are observed frequently in 46,XY disorders of sex development (DSD) with normal adrenal function (Köhler B. *et al*. 2008). The SF1 mutations results in the phenotypic spectrum ranges from complete testicular dysgenesis with Mullerian structures, through individuals with mild clitoromegaly or genital ambiguity, to severe penoscrotal hypospadias or even anorchia (Lin L *et al*. 2008). SF1 probably regulates enzyme mediating steps in steroid formation as well as the transcription of AMH (Ingraham HA *et al*. 1994).

WT1 has an essential role in urogenital development (Parker K.L *et al*. 1999, Dewing P *et al*. 2002). Abnormalities in the WT1 gene are associated with failure of gonadal differentiation, nephropathy, development of Wilms tumors (in Denys-Drash syndrome),

gonadoblastoma (in Frasier syndrome) (Hiort O *et al*. 2000). The role of WT1 gene in gonadal differentiation is not sex specific, as gonadal dysgenesis also occurs in 46,XX individuals (Rey R *et al*. 1998, Nachtigal MW *et al*. 1998).

Lim1 expression in the Müllerian duct is dynamic, corresponding to its formation and differentiation in females and regression in males. Lim1 is essential for the female reproductive tract development (Kobayashi, A *et al*. 2004). LIM1-gene is involved in the development of the bipotential gonand and the kidneys (Lim HN *et al*. 1998). Homozygous mutations in this region lead to the failure of both gonads and kidneys in mice. So far no human mutations have been described in this gene (Hiort O *et al*. 2000).

RSPO1 is a novel regulator of the Wnt/beta-catenin signalling pathway (Smith CA *et al*. 2008). RSPO1 leads to complete female-to-male sex reversal in the absence of the testis determining gene, SRY (Parma R *et al*. 2006). Rspo1 null mutant XX mice are masculinized with depleted germ cells, male-like vascularisation, deregulation of Wnt4 expression and ectopic testosterone production (Chassot AA *et al*. 2008, Tomizuka K *et al*. 2008). RSPO1 could be a key ovary determining gene in humans and mammals (Wilhelm D *et al*. 2007, Parma R *et al*. 2006). RSPO1 is an important ovarian development gene in amniotic vertebrates (Smith CA *et al*. 2008).

46,XY complete pure gonadal dysgenesis (PGD) is not only caused by SRY gene mutations but also by mutations of the DHH gene (Canto P *et al*. 2004).

DMRT1 is essential for testis differentiation (Raymond CS *et al*. 2000). DMRT1 and 2 deletions lead to 46,XY gonadal dysgenesis (Ottolenghi C *et al*. 2000). Dmrt1 is expressed in both Sertoli cells and germ cells. Dmrt1 is required for Sertoli cell differentiation and for germ cell survival (Kim *et al*. 2007b). Dmrt1 is required in germ cells for radial migration and survival of gonocytes. Dmrt2 is essential for somite development (Seo KW *et al*. 2006). Dmrt genes not only plays a major role in testis determination in mammals but also appears to be involved in other developmental processes (Hong C.S *et al*. 2007).

1.2.3 Hormonal control of fetal sex development

Fetal development of the female phenotype does not require estrogens. In contrast, male sexual differentiation requires high concentrations of androgens. The main androgens are testosterone (T) and dihydrotestosterone (DHT). Initially androgens are synthesized autonomously, later on under the control of placental human chorionic gonadotropin (hCG) secretion. Later in gestation, androgen synthesis is controlled by luteinizing hormone (LH) secretion from the pituitary gland (Hughes I.A *et al*. 2002).

Testosterone secreted by the Leydig cells around 8 weeks of gestation in the 46,XY embryo causes differentiation of the Wolffian ducts into the male internal genitalia (the epididymis, vas deferens and the seminal vesicles) (Figure 4). Testosterone is converted into DHT with the help of the 5α-reductase type II enzyme in the androgen target tissues. DHT influences the virilization of the external genitalia (male urethra, prostate, penis and scrotum) and plays a major role in the development of secondary sexual characteristics at male puberty (Wilson, J.D *et al*. 1978).

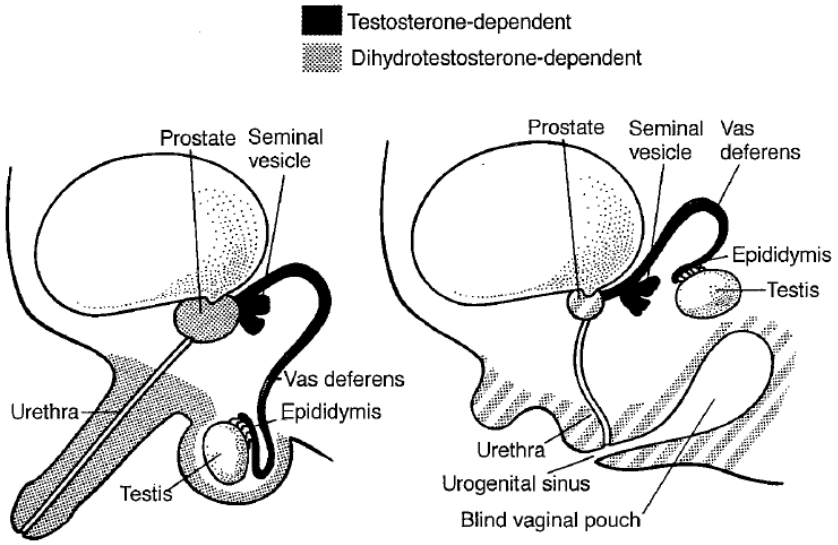

Figure 4. Illustration of the androgen dependent development in the male sexual differentiation in utero (Zhu Y-S *et al*. 1998).

1.2.4 Androgen receptor structure and function

AR is a steroid hormone receptor and belongs to the ligand –activated subgroup of nuclear receptors (NR) superfamily (Fuller PJ *et al*. 1991, Mangelsdorf DJ *et al*. 1995, Beato M *et al*. 2000). The AR gene consists of eight exons and is located on the long arm of the X chromosome (Xq11-12) (Brown CJ *et al*. 1989). The exons are designated as A-H or 1-8. AR is approximately 90 kilobases (kb) long and encodes a protein of approximately 110-kDa (919 amino acids) (Lubahn DB *et al*. 1989). AR consists of separate domains namely, the N-terminal, transcription activation domain (NTD, (exon 1)) preceding a DNA binding domain (DBD), followed by a hinge region and a carboxy-terminal region containing the ligand binding domain (LBD) (Figure 5) (Jenster G *et al*. 1991). The DBD (exon 2 and 3) consists of 68-amino acid region and folds into two zinc finger structures to be able to bind to DNA. The 295-amino acid region behind the DBD, including the hinge region and the LBD (exon 4 to 8), is responsible for dimerization and androgen binding (Sack JS *et al*. 2001). The nuclear localization signal (NLS) is responsible for nuclear trafficking of AR (Baumann CT *et al*. 1999). NLSs are usually located in flexible regions of proteins (Stewart M 2007). The putative AR bipartite NLS (residues 617-620) lies within the terminal α-helix of the second zinc finger and not within the flexible hinge region (Cutress ML *et al*. 2008). Both structural and functional studies support that the NTD of AR consists of a region called AF-1 (141 to 338 amino acids) which is involved in transcriptional activation (Simental JA *et al*. 1991). Transactivation domains AF-2 in the LBD (Berrevoets CA *et al*. 1998) and AF-5 (369 to 493 amino acids) have been mapped in the NTD (Brinkmann AO *et al*. 2001).

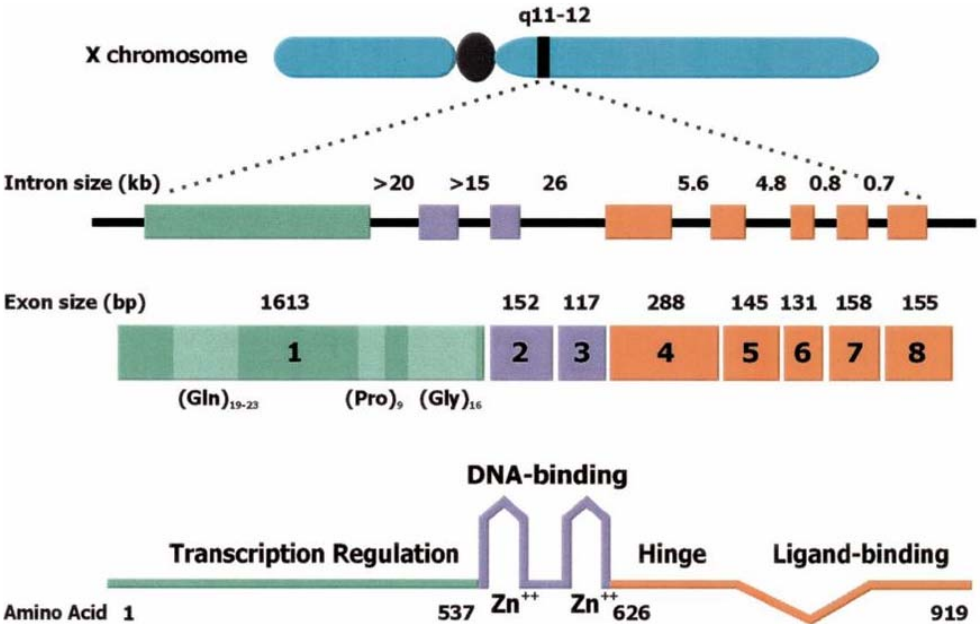

Figure 5. Schematic illustration of the AR gene, protein. The AR gene spans more than 80 kb that includes the exonic organization shown in the second panel. Location of the three codon repeat regions in the first exon that codes for the N-terminal domain is shown in the third panel. The diagram of the protein structure demonstrates translation of exons into discrete functional regions of the receptor (Gelmann EP *et al*. 2002).

The androgen-AR complex can either induce or repress androgen responsive genes by binding to specific DNA sequences called hormone response elements (HRE), also known as androgen response elements (ARE) (Chang C *et al*. 1995, Chang C *et al*. 1992) (Figure 6). Testosterone (T) and Dihydrotestosterone (DHT) are the most important ligands of the AR (Hsiao PW *et al*. 2000). Methyltrienolone (R1881), a synthetic ligand of AR has been used for experimental purposes (Matias PM *et al*. 2000).

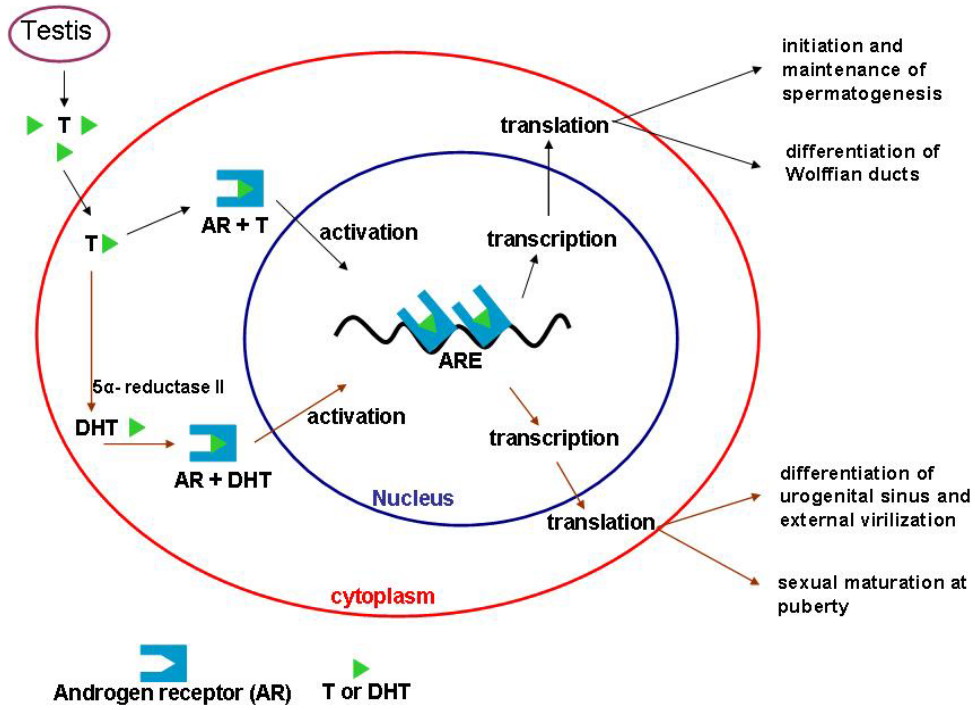

Figure 6. Molecular mechanism of AR action. Testosterone (T) enters the target cell (by passive diffusion) (not shown in the picture)) and is either binding directly to the androgen receptor (AR) or is metabolized to Dihydrotestosterone (DHT) and binds to the AR. Both T or DHT can bind to the same AR. T and DHT have their individual roles in male sexual differentiation.

The AR is a key factor of normal male sexual differentiation and is crucial for functional maintenance of the male reproductive tissues. During puberty and adulthood, the AR mediates a wide variety of different biological effects in various tissues including beard growth, deepening of the voice, growth spurt, increase of the body muscle mass contributing to the typical male body shape, acne, and male pattern baldness. AR effects the development and the function of diverse organ systems and tissue types including bone (Vanderschueren D *et al*. 2004), bone marrow (Claustres M *et al*. 1998), liver (Cohen C *et al*. 1998), brain (Dörner G *et al*. 2001), and certain aspects of gender (Meyer-Bahlburg HF *et al*. 2004). Moreover, AR plays important roles in pathological conditions like benign prostatic hyperplasia (Andriole G *et al*. 2004) and prostate cancer (Culig Z *et al*. 2003).

1.3 Disorders of Sex development (DSD)

DSD refers to any phenotype in which a specific sex can not be assessed. Previously it was called intersex, hermaphrodite and pseudohermaphrodite. It is difficult to define DSD as it covers many different aspects like classical phenotype and the biological state. This new nomenclature-DSD, uses karyotype as a prefix for example- XY DSD or XX DSD, respectively. Immediate sex assignment after birth in patients with DSD is not possible as it requires multidisciplinary professionals. Table 1 gives the revised classification of DSD (Hughes I.A 2008). The XX DSD and XY DSD were subdivided according to the primary disorder of gonad development versus a disorder of sex steroid synthesis arising from an otherwise morphologically normal gonad.

Table 1. A proposed classification of DSD

Sex chromosome DSD	46,XY DSD	46,XX DSD
A: 47,XXY Klinefelter Syndrome und Variants **B: 45,X** Turner Syndrome und Variants **C: 45,X/46XY Mosaic** Mixed gonadal dysgenesis **D: 46,XX/46XY** Chimerism	**A: Disorders of gonadal (testicular) development** 1. Complete or partial gonadal dysgenesis (e.g. SRY, SOX9, SF1, WT1, DHH etc) 2. Ovotesticular DSD 3. Testis regression	**A: Disorders of gonadal (ovarian) development** 1. gonadal dysgenesis 2. Ovotesticular DSD 3. Testicular DSD (e.g. SRY+, dup SOX9, RSP01)
	B: Disorders in androgen synthesis or action	**B: Androgen excess**
	<u>Disorders of androgen synthesis</u> LH receptor mutations Smith-Lemli-Optiz syndrome Steroidogenic acute regulatory protein mutations Cholesterol side-chain cleavage (CYP11A1) 3ß-hydroxysteroid dehydrogenase 2 (HSD3ß2) 17α-hydroxylase/17,20-lyase (CYP17) P450 oxidoreductase (POR) 17ß-hydroxysteroid dehydrogenase (HSD17ß3) 5α-reductase 2 (SRD5A2)	<u>Fetal</u> - 3ß-hydroxysteroid-dehydrogenase Typ II (HSD3ß2) - 21-hydroxylase (CYP21A2) - P450 oxidoreduktase (POR) - 11ß-hydroxylase (CYP11B1) - Glucocorticoid receptor mutaions <u>Fetoplacental</u> - Aromatase deficiency (CYP19) - P450 Oxidoreductase deficiency (POR) <u>Maternal</u> - Virilizing tumors (e.g. luteomas) - Androgenic drugs
	<u>Disorders of androgen action</u> Androgen Insensitivity syndrome (AIS) Drugs and environmental modulators	
	C: Other 1. Syndromic associations of male genital development (e.g. cloacal anomalies, Robinow, Aarskog, Hand-Foot-Genital, popliteal pterygium) 2. Persistent Müllerian duct syndrome 3. Vanishing testis syndrome 4. Isolated hypospadias (CXorf6) 5. Congenital hypogonadotropic hypogonadism 6. Cryptorchidism (INSL3, GREAT) 7. Environmental influences	**C: Other** 1. Syndromic associations (e.g. cloacal anomalies) 2. Müllerian agenesis/ hypoplasia e.g. MURCS) 3. Uterine abnormalities (e.g. MODY5) 4. Vaginal atresis (e.g. KcKusickeKaufman) 5. Labial adhesions

1.3.1 46,XX DSD

1.3.2 Congenital adrenal hyperplasia (CAH)

CAH is the most common cause of 46,XX DSD. 90–95% of CAH cases are caused by 21-hydroxylase deficiency. Females affected with severe, classic 21-hydroxylase deficiency are exposed to excess androgens prenatally and are born with virilized external genitalia (White PC *et al*. 2000, Hughes I.A. 2002). These individuals can not synthesize cortisol. Inefficient cortisol synthesis signals the hypothalamus and pituitary to increase Corticotropin-releasing hormone (CRH) and Adrenocorticotropic hormone (ACTH), respectively. Consequently, the adrenal glands become hyperplastic. But rather than cortisol, the adrenals produce excess sex hormone precursors that do not require 21-hydroxylation for their synthesis. Once secreted, these hormones are further metabolized to active androgens-testosterone and dihydrotestosterone-and to a lesser extent estrogens-estrone and estradiol. The net effect is prenatal virilization of girls and rapid somatic growth with early epiphyseal fusion in both sexes. About three-quarters of patients cannot synthesize sufficient aldosterone to maintain sodium balance and are termed 'salt wasters'. This predisposes them to episodically develop potentially life-threatening hyponatremic dehydration.

1.3.3 Placental aromatase deficiency (CYP19)

The P450 enzyme aromatase (CYP19) plays a crucial role in the endocrine and paracrine biosynthesis of estrogens from androgens in diverse estrogen-responsive tissues. Complete aromatase deficiency has been reported in a small number of 46,XX girls with genital ambiguity and absent pubertal development (Lin L *et al*. 2007).

1.3.4 Cytochrome P450 oxidoreductase (POR)

It has been recently added to the list of 46,XX DSD (Hughes I.A, 2008). POR deficiency is listed in both 46,XX DSD and 46,XY DSD (Table 1). Microsomal P450 enzymes metabolize drugs and catalyze steroid biosynthesis. POR knockout mice are embryonically lethal. Recessive human POR missense mutations causes disordered steroidogenesis and Antley-Bixler syndrome (ABS) (Miller W.L *et al*. 2005).

1.3.5 46,XY DSD

The following disorders belongs to 46,XY DSD: Leydig cell hypoplasia, 17ß-HSD type III defect, Gonadal dysgenesis due to the mutations of SRY, SF1, WT1, gonadal dysgenesis due to deletion of DMRT1, Persistant Mullerian duct syndrome and Androgen insensitivity syndrome (AIS).

1.3.6 Leydig cell hypoplasia

Leydig cell differentiation is crucial for adequate androgen production by the testis. Leydig cell hypoplasia is a rare autosomal recessive condition caused by mutations in the LH receptor gene. It interferes with normal development of male external genitalia in 46,XY individuals due to defective luteinizing hormone (LH) receptor signal transduction (Kremer H *et al*. 1995, Richter-Unruh A *et al*. 2002). The phenotype ranges from complete female external genitalia to males with micropenis (Zenteno JC *et al*. 1999)

1.3.7 17ß-HSDIII deficiency- an autosomal recessive form of male sex reversal

17ß-hydroxysteroid dehydrogenase (17ß-HSD) III accounts for a defect in testosterone biosynthesis from androstenedione (Figure 7). In fetal testes, defects in the conversion of androstenedione to testosterone by the enzyme 17ß-HSDIII give rise to genetic males with female external genitalia (Geissler WM *et al*. 1994). The phenotype of 17ß-HSDIII deficient males is similar to that of 5α-reductase 2 deficiency, both have male Wolffian-duct derived internal genitalia and external genitalia that are almost always female in characteristics. However, 17ß-HSDIII deficiency is distinguished from 5α-reductase 2 deficiency by elevated serum levels of androstenedione, the substrate of the 17ß-HSDIII enzyme.

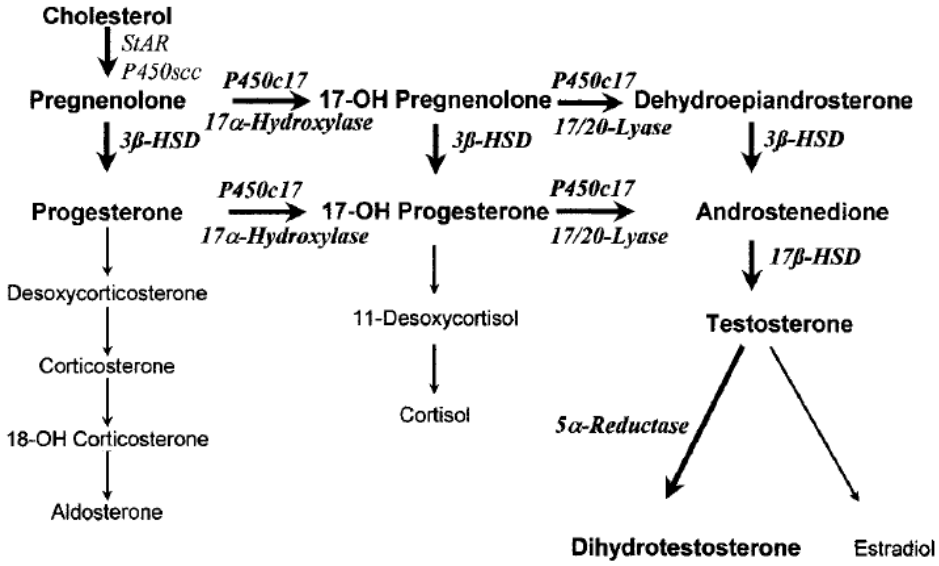

Figure 7. Pathways and metabolites of androgen biosynthesis (Hiort O *et al*. 2000).

1.3.8 5α-reductase type II deficiency

The conversion of T to DHT by 5α-reductase type II is essential for the development of male external genitalia. In general, affected individuals can vary from female phenotype at birth to isolated hypospadias. However, newborns with 5α-reductase type II deficiency can have severe sexual ambiguity with a clitoral-like phallus, severely bifid, generally empty scrotum, and pseudovaginal perineoscrotal hypospadias. A urogenital sinus with a blind vaginal pouch opens into the perineum. Wolffian duct differentiation is normal with seminal vesicles, vas deferens, and epididymis, and the prostate is undeveloped. There are no Müllerian structures (Sultan C *et al*. 2002).

1.3.9 Androgen insensitivity syndrome (AIS)

Mutations in the X-chromosomal AR-gene inhibit AR-function and in turn lead to AIS in 46,XY individuals (Quigley CA *et al*. 1995, Deeb A *et al*. 2005). The AR gene mutation database has over 500 reported mutations. The database is available on the internet at http://.mcgill.ca/androgendb/(EMBL-European Bioinformatics Institute (ftp.ebi.ac.uk/pub/databases/androgen). The clinical spectrum of AIS ranges from complete AIS (CAIS) with normal female external genitalia due to complete inactivation of the AR to partial AIS (PAIS) with varying degrees of genital ambiguity including clitoromegaly or

labial fusion to unambiguously male with undervirilization such as hypospadias or micropenis, slight defects of virilization or isolated infertility caused by partial insufficiency of AR-function (Figure 8) (Quigley CA *et al*. 1995, Deeb A *et al*. 2005). The main phenotypic characteristics of CAIS are female external genitalia, consisting of a short, blind ending vagina, the absence of Wolffian duct derived structures, the absence of prostate, development of breast and the absence of pubic and axillary hair (Quigley CA *et al*. 1995). Usually testosterone levels are elevated at the time of puberty, while also elevated LH levels are found. At puberty, elevated LH, testosterone and estradiol levels are observed. Usually, testosterone levels are elevated at the time of puberty, while also elevated LH levels are found indicating androgen resistance at the hypothalamic-pituitary level. The high testosterone aromatizes into estrogens, which are responsible for further feminization in CAIS individuals.

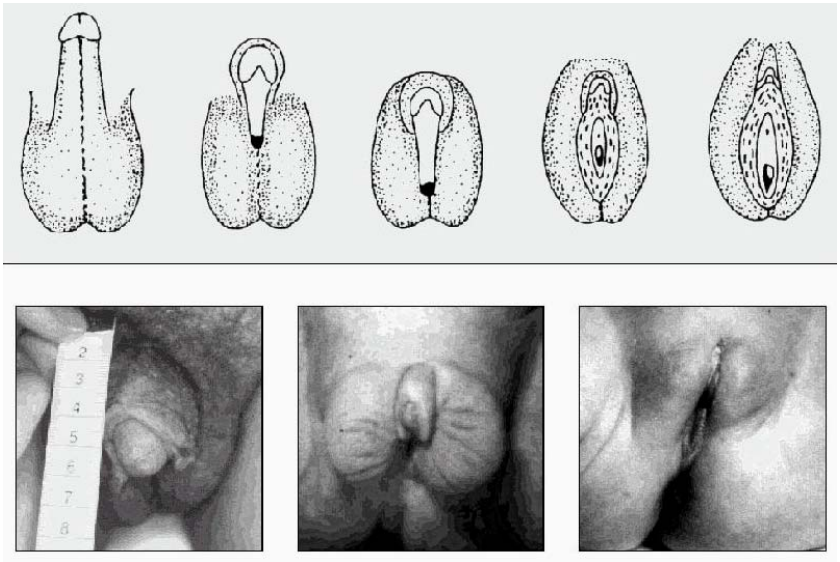

Figure 8. Photos and cartoons illustrating the androgen insensitivity syndrome

Unresolved questions in AIS (molecular diagnosis)

Many patients with clinically evident AIS do not have documented mutations in the AR gene (Deeb A *et al*. 2005). These individuals might have defects in yet uncharacterized coregulators of AR signaling. The interaction of the AR with specific coregulatory proteins is a potential mechanism for modulating specificity of androgen actions. Coregulators are proteins that interact with nuclear receptors to enhance transactivation (coactivators) or reduce transactivation (corepressors) (Heinlein CA *et al*. 2002). *In vitro* studies have demonstrated

that the transcriptional activity of the AR can be significantly altered by coregulators (Heinlein CA *et al*. 2002, Hong H *et al*. 1997). For instance, there is evidence that co-regulators such as transcriptional intermediary factor 2 (TIF2) are involved in the pathogenesis of AIS (Ghali SA *et al*. 2003, Quigley CA *et al*. 2004, Umar A *et al*. 2005). An AR coactivator defect has been suggested to be the sole cause of AIS in the absence of an AR gene mutation (Adachi M *et al*. 2000).

In some patients, reduced AR transcription due to unknown mechanisms may also underlie AIS (Holterhus PM *et al*. 2005). These considerations hamper clinical management due to the lack of a defined molecular diagnosis and clinical prognosis in a significant subset of patients. Moreover, in AIS patients with an established AR gene mutation, the correlation between genotype and phenotype correlation is often poor, even within the same family (Figure 9) (Rodien P *et al*. 1996, Evans BA *et al*. 1997, Holterhus PM *et al*. 2000). Therefore, the discovery of transcriptional biomarkers could provide opportunities to develop a bioassay for the diagnosis of AIS based on functional criteria.

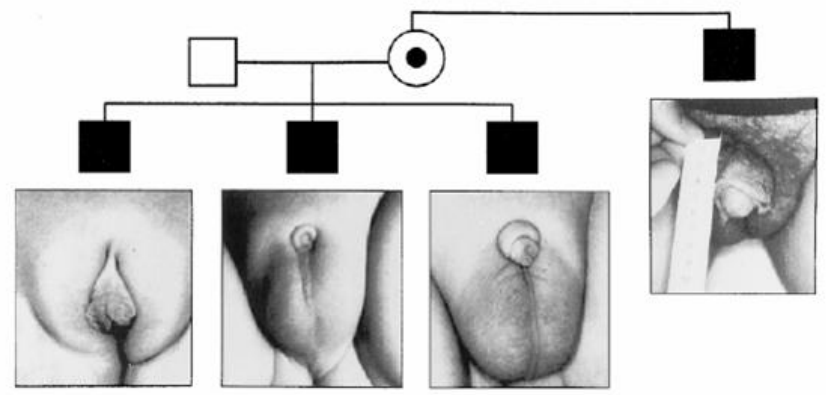

Figure 9. Pedigree of the family of this study and respective phenotypes. From left to right, brother 1 (B1), brother 2 (B2), brother 3 (B3), and their uncle. (Holterhus PM *et al*. 2000).

1.4 Genital skin fibroblasts (GSF) as a model of identifying androgen regulated genes

Historically, the clinical diagnosis AIS was confirmed by reduced affinity of binding of androgen to the AR in cultured genital skin fibroblasts. However, this method is restricted to patients having molecular defects in the ligand binding domain of the AR (McPhaul MJ *et al*. 1993). The degree of AR dysfunction in AIS also used to be assessed by the degree of decrease of Steroid Hormone Binding Globulin in response to Stanozolol (Sinnecker G *et al*. 1989, Sinnecker GH *et al*. 1997). However, it is unclear whether this test reflects the competence of the AR as a transcriptional regulator, making test results difficult to interpret. Attempts to identify androgen regulated genes in AR expressing male foreskin fibroblasts - a commonly used "normal control" in human genital differentiation research - either failed or showed inconsistent results (Berkovitz GD *et al*. 1990, Stillman SC *et al*. 1991, Nitsche EM *et al*. 1996, Holterhus PM *et al*. 2003, Bebermeier JH *et al*. 2006). Partly, these inconsistencies may be explained by the fact that post natal genital fibroblasts represent end-stage differentiated cells that have become unresponsive to androgen signaling since genital morphogenesis has been completed (Holterhus PM *et al*. 2003, Bebermeier JH *et al*. 2006). It has been recently shown that skin fibroblasts derived from the scrotum, which is the male homologue of the labia majora in females, display significant differences of gene expression patterns compared with foreskin fibroblasts indicating that these two cell types are very different entities (Holterhus PM *et al*. 2007).

1.5 Peripheral blood mononuclear cells (PBMCs) as a model of identifying androgen regulated genes

Peripheral blood has been an attractive tissue type for biomedical and clinical research because of its critical role in immune response metabolism and ease of collection. Blood is a valuable resource for biomarker discovery and development of diagnostics in hematological and non-hematological diseases. Blood gene expression profiling for disease classification and diagnosis were successful in acute lymphoblastic leukemia (ALL), acute chronic hepatitis B infection large B cell lymphoma, chronic fatigue syndrome, lung cancer, aggressive periodontitis and chronic arthritis. (Fan H *et al*. 2005, Trehanpati N *et al*. 2008, Sorensen B *et al*. 2009, Liu L *et al*. 2008, Sørensen LK *et al*. 2008). Peripheral blood can also be used in the

investigation of pharmacodynamic effects of drugs at a genomic level to predict clinical outcome, efficacy and also side effects.

Blood pathophysiology (components in blood)

Blood consists of multiple cells at different growth stages, such as White blood cells (WBCs), Red blood cells (RBCs), platelets and plasma.

WBC

WBCs constitute less than 1% of the blood volume. WBCs consist of about (30%) lymphocytes, (5%) Monocytes and (65%) granulocytes. The lymphocytes are subdevided into T and B lymphocytes. Graulocytes are comprised of neutrophils (95%), eosinophils (4%) and basophils (1%). Peripheral blood mononuclear cells (PBMCs) contain monocytes and lymphocytes. PBMCs are the most transcriptionally active cells in the blood. Thus, most studies in the biomarker research prefer the use of PBMCs.

RBC

RBC lose their nucleus and organelles during their differentiation. RBC are transcriptionally inactive as they do not contain a nucleus. Thus, they can not be used as a model for gene expression studies. Although reticulocytes (immature RBC) contain RNA, they are often transcriptionally inactive.

Plasma, Platelets

Plasma makes up about 55% of the blood. It consists of 90% water and other dissolved substances. *Platelets* are tiny cellular elements produced by bone marrow. Platelets do not have nuclei and contain minute amounts of translationally active mRNA. Clinical use of platelets is often limited (especially in pediatric patients) due to the low yield of megakaryocytic-derived mRNA and possible contamination with leukocytes (Schedel A *et al*. 2009). Platelet transcriptome will be useful for identifying proteins that regulate normal and pathologic platelet functions (Gnatenko DV *et al*. 2003). The potential application of platelet specific microarrays in clinical settings are cardiovascular and cerebrovascular diseases (Bahou WF *et al*. 2004).

Androgens (mainly T) are synthesized and secreted into the blood stream (Hsiao PW *et al*. 2000). Androgen binding sites were identified in human peripheral mononuclear leukocytes of healthy males and females by using (^{3}H) dihydrotestosterone, RU-1881 and (^{3}H) testosterone (Kuhnle U *et al*. 1994). This group used the androgen binding assays to prove the experimental evidence of AR expression in blood leukocytes (Kuhnle U *et al*. 1994). The combined treatment of dehydroepiandrosterone (DHEA) and lipopolysaccharide (LPS) on human monocytes release interleukin-6 (IL-6) (Delpedro AD *et al*. 1998). Peripheral monocytes produce the interleukin-1 (IL-1) in presence of progesterone or 17-ß estradiol (estradiol), and testosterone (Morishita M *et al*. 1999). Sex steroid metabolism in PBMCs changes significantly with age (Hammer F *et al*. 2005).

1.6 Microarray technology

Gene expression tools in molecular biology have advanced from analyzing one gene or gene pathway at a time to whole genome-wide pathways with the help of microarrays. Microarrays are similar to Northern-blotting analysis, but the target or labeled RNA (genome-wide) binds to immobilized cDNA or oligo nucleotides (complementary gene sequences) printed on the surface of microscopic slide. Microarray technology is advancing rapidly in terms of surface chemistry used to bind the cDNAs or Oligo nucleotides on a glass slide, new labeling protocols, new dyes and increasing number of genome sequences in other organisms.

1.7 Objectives of the project

Genome-wide screening of biomarkers of transcriptional AR function in Androgen insensitivity syndrome (AIS) by using primary genital skin fibroblasts (GSF) obtained from healthy, patients, patients with molecular proven AIS and PBMCs from healthy donors.

1) Identification of a model GSF for detecting AR transcriptional regulation in AIS.

2) Genome-wide screening of androgen induced genes in normal scrotum compared to 46,XY labia majora fibroblasts from patients with complete AIS.

3) Quantitative RT-PCR based reconfirmation of androgen induced genes in independent cell strains derived from AIS patients with different phenotypes and different AR gene mutations.

4) Quantitative RT-PCR based analysis of androgen induced genes in XY labia majora fibroblasts from patients with defects in androgen biosynthesis.

5) Genome-wide screening of androgen induced genes in PBMCs treated *in vitro*.

2 Materials and Methods

2.1 Chemicals, reagents, buffers and kits

Products	Company	Cat#
APOD (Apolipoprotein D) primers	TIB Molbiol	for (no.11). 874497 rev (no.11). 874498
SDHA primers (Succinate dehydrogenase complex, subunit A)	TIB Molbiol	for 874499 rev 874500
AZA (5'-aza-deoxy-cytidine)	Sigma-Aldrich	A3656
BSA (bovine serum albumin)	Sigma-Aldrich	B4287-5G
Chloroform	J.T. Becker	67-66-3
Cy3, Cy5 dyes	Amersham	PA55322
Cot-1 human DNA	Invitrogen	15279-011
dNTPs	Amersham	27-2035-02
DMEM (Phenol red-free) (Dulbecco's modified Eagle's Medium)	Gibco-BRL	31053-028
Ethanol	J.T. Becker	64-17-5
EDTA (pH 8.0) (Ethylene-diamine-tetra-acetic acid)	Flucka	03690
Ficoll reagent	Biochrom AG	L6115
FBS (fetal bovine serum)	Biochrom AG	S0113
HEPES (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid)	Gibco-BRL	15630
HCl (Hydrochloric acid)	J.T. Becker	7647-01-0
Pencillin/ Sterptomycin	Sigma	P0781
Poly A RNA	Invitrogen	
RNase ZAP	Ambion	9780

Recovery cell culture medium (cell culture freezing medium)	Gibco	12648-010
L-Glutamine	Biochrom AG	K0283
NaOH	J. T.Becker	1310-73-2
Yeast tRNA	Invitrogen	15401-011
SSC buffer	Flucka	93017
SDS buffer	Sigma	L4522
SuperScript II	Invitrogen	18064-071
Trizol	Invitrogen	115596-018
TE (Tris EDTA, pH 7.4) buffer	Flucka	93302
Trypsin-EDTA (10x)	Sigma	T4174
Universal human reference RNA	Stratategene	740000
X-vivo cell culture medium	Cambrex	BE04-418Q
1x Dulbecco's PBS (phosphate-buffered saline)	PAA	H15-002

Kits	**Company**	**Cat#**
Coverslips (microarrays)	Erie Scientific Co.	25X601-2-4789
Chip priming station (RNA analysis)	Agilent	5065-4401
First strand cDNA synthesis kit	Fermentas	K1622
QuantiTech SYBR green PCR kit	Qiagen	1970795
RNeasy mini kit	Qiagen	74104
DNA-free Kit	Ambion	AM1906
Fragementation reagents (oligo arrays)	Ambion	AM8740
RNA Nano Chip	Agilent	5065-4413
RNA 6000 reagents	Agilent	5067-1512
RNA ladder (nano chip)	Agilent	5067-1529

Microarrays Human Exonic Evidence Based Oligonucleotide (HEEBO) array Human cDNA microarrays	Stanford functional genomics facility (SFGF)	
Microcon YM-30 columns	Millipore	42410
MessageAmpTM II aRNA kit (cDNA arrays)	Ambion	AM1751
MessageAmpTM II aRNA kit (Oligo arrays)	Ambion	AM1753

Instruments	**Company**
Centrifuge (for Eppendorfs)	Eppendorf 5415D
Centrifuge (for microarray slides)	Beckman Coulter Allegra^{TM} X-22R
Centrifuge (for PBMCs separation)	Heraeus Multifuge 3 SF
Hot plate	Techne Dri-Block DB-1
Genepix 4000B microarray scanner	Axon, USA
Incubator	Heraeus
Light cycler	Roche Diagnostics
Millipore water	Millipore synergy 185
PCR	Eppendorf
UV cross linker	Amersham Life Sciences, USA
Vortex	VWR international
Vaccum drier	Christ RVC 2-18
Water bath	Gfl
2100 Bioanalyzer	Agilent

2.2 Experimental subjects

The study was approved by the ethical committee of the University of Lübeck, Germany. A written informed consent was obtained from normal subjects, patients and their parents.

2.3 Fibroblast strains, cell culture

The fibroblast strains were characterized by using androgen binding assays (Table 2). Scrotum skin fibroblasts were obtained from 8 males with a phenotypically normal male external genitalia. 46,XY labia majora and labioscrotal derived fibroblasts were obtained from 6 PAIS – and 5 CAIS patients with proven inactivating mutations of the AR gene. In addition 2 labia majora derived fibroblast cultures with 17ß-HSDIII deficiency due to documented inactivating mutations were analyzed (Table 2). Fibroblasts were grown at 37°C with 5% CO_2 and 90% humidity. They were cultured in phenol red free DMEM (Gibco BRL, Germany) supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM L-Glutamine (Sigma, St. Louis, MO), and 20 mM HEPES buffer (Gibco BRL, Eggenstein, Germany). All fibroblast strains grew slowly and were highly sensitive to mechanical disruptions like harsh pipetting and enzymatic digestions like trypsin. All hormone treatments were carried out with the above mentioned medium constituents but with charcoal treated 10% FBS.

Table 2. List of fibroblast strains

Fibroblast strains; ARD, patient strain-ID; S, normal male scrotal fibroblasts. Normal ranges for androgen (methyltrienolone) binding: Bmax (binding capacity): 13-116 fmol/mg protein; Kd (dissociation constant): 0.03-0.13 nM.

cell strain / *passage number*	**anatomic origin / *embryonal anlagen***	**anatomic phenotype of external genitalia /** *AIS-stage*	**age at biopsy years; months**	**selected clinical background and functional data (androgen binding, AR-genotype, specific remarks)**
S 3 *3*	scrotum, right side *labioscrotal swellings*	normal male	12;7	Torsion of left testis 4 weeks ago, clinical indication for surgery: prophylactic orchidopexy on the right side, no androgen binding data
S 4 *6*	scrotum, left side *labioscrotal swellings*	normal male	1;3	Minor deviation of penile shaft but no hypospadias, maldescensus testis on both sides, indication for surgery: orchidopexy; K_d 0.12 nM, B_{max} 44 fmol/mg protein
S 5 *8*	scrotum, right side *labioscrotal swellings*	normal male	1;7	Suspected inguinal testis on the right side. Clinical indication for surgery: orchidopexy, upon surgery no testicular tissue present on the right, K_d 0.09 nM, B_{max} 28 fmol/mg protein

S 8 *9*	scrotum, raphe *labioscrotal swellings*	normal male	31;0	Infertility, azoospermia, clinical indication for surgery: testicular sperm extraction, hormones before biopsy: LH = 3.5 U/L, FSH = 2.7 U/L; testosterone = 276.31 mg/dl; fibroblast genomic DNA sequence analysis upon knowledge of APOD RT-PCR revealed I841S point mutation within the ligand binding domain of AR
S 9 *7*	scrotum, raphe *labioscrotal swellings*	normal male	34;0	Infertility, azoospermia, clinical indication for surgery: testicular sperm extraction; K_d 0.08 nM, B_{max} 55 fmol/mg protein
S 12 *9*	scrotum, left side *labioscrotal swellings*	normal male	9;2	Maldescensus testis on the left side, clinical indication for surgery: orchidopexy on the left, K_d 0.07 nM, B_{max} 37.09 fmol/mg protein
S 13 *5*	scrotum, raphe *labioscrotal swellings*	normal male	58;1	Infertility, azoospermia; clinical indication for surgery: testicular sperm extraction; no androgen binding data.
S 15 *7*	scrotum, raphe *labioscrotal swellings*	normal male	5;2	Phimosis; clinical indication for surgery: circumcision; no androgen binding data
ARD 446 *6*	scrotum *labioscrotal swellings*	predominantly male (AIS2)	7;8	silent mutation GGG>GGA in codon 795 of the AR-gene; K_d 0.07 nM, B_{max} 26 fmol/mg protein
ARD 001 *3*	labia majora/ scrotum *labioscrotal swellings*	ambiguous (AIS3)	0;9	exonic splice site mutation AGC>AGT in codon 888 leading to aberrant splicing.

ARD 084 *9*	labia majora/ scrotum *labioscrotal swellings*	ambiguous (AIS3)	0;8	no mutation in the whole coding region of the AR gene, reduced AR-mRNA, reduced AR-protein and reduced ligand binding (K_d 0.05 nM, B_{max} 6 fmol/mg protein)
ARD 534 *10*	labia majora/ scrotum *labioscrotal swellings*	ambiguous (AIS3)	2;4	Arg608Lys
ARD 377 *4*	labia majora/scrotum *labioscrotal swellings*	predominantly female (AIS4)	1;2	Ile841Ser; K_d 0.55 nM, B_{max} 17 fmol/mg protein
ARD 659 *4*	labia majora *labioscrotal swellings*	predominantly female (AIS4)	3;10	Ala870Gly
ARD 402	labia majora *labioscrotal swellings*	normal female (CAIS)	1;0	2 base pair deletion in exon1, frameshift, premature stop codon, negative androgen binding, very low AR-mRNA transcription, no AR-protein in Western immunoblot
ARD 411 *7*	labia majora *labioscrotal swellings*	normal female (CAIS)	0;4	Arg855Cys, negative androgen binding
ARD 682 *8*	labia majora *labioscrotal swellings*	normal female (CAIS)	14;10	Gln59stop, negative androgen binding, no AR-protein in western blotting
ARD 1097 *4*	labia majora *labioscrotal swellings*	normal female (CAIS)	1;3	Pro390Ser + Arg855Gly; negative androgen binding
ARD 1144 *4*	labia majora *labioscrotal swellings*	normal female (CAIS)	4;3	pathological androgen binding, K_d 1.59 nM, B_{max} 14 fmol/mg protein, no mutation detected

ARD 111 *7*	labia majora *labioscrotal swellings*	normal female	10;9	Homozygous disruptive mutation 325+4A/T of the 17ß-HSDIII gene
ARD 1373 *9*	labia majora *labioscrotal swellings*	normal female	13;4	Homozygous disruptive mutation 325+4A/T of the 17ß-HSDIII gene

2.3.1 Sub-culturing, Freeze-Thawing of fibroblasts

The medium from the confluent cells was aspirated and the cells were washed with 1x PBS. The PBS was removed and the cells from the confluent dish were separated with 3 ml of 0.25 Trypsin-EDTA solution (Sigma, Germany) at 37°C for 4 min. Trypsin was neutralized with 7 ml of DMEM containing serum and the cells were centrifuged at 1000 rpm for 3 min. The supernatant was discarded and the cells were resuspended in 1 ml of cold (4°C) freezing medium (Recovery cell culture medium, Gibco). The cells were resuspended carefully by pipetting up and down without applying extra pressure. The cell suspension was transferred to cryovials (Nunclon) and frozen in a cell freezing box (Nalgene) at -80°C. The next day cryovials were stored in liquid nitrogen.

For thawing, cryovials were kept at 37°C water bath until thawed. The cell suspension was transferred into a 15 ml falcon tube containing 10 ml DMEM (supplemented with 10% charcoal treated FBS + 100 U/ml pencillin/streptomycin, 2 mM Glutamine, 20 mM HEPES). Cells were centrifuged at 1000 rpm for 3 min. The supernatant was removed, cells were resuspended carefully in 1 ml DMEM, to this additional 3 ml of the DMEM were added. The cell suspension with medium was transferred to T-175 flasks with 20 ml DMEM.

2.3.2 Counting of the cells with hemocytometer

The cells were counted using a *Neubauer* hemocytometer. The chamber of the hemocytometer was filled with the cell suspension by capillary action. The total number of cells in the four marked squares were counted using a microscope. A cover glass was placed over these squares.

The number of cells were estimated by using the following formula

Number of cells/ ml= (cells in 4 large squares/4) x dilution factor x 10^4

2.3.3 Culturing and treating the cells with hormones

In the first set of experiments, two different scrotum fibroblasts strains (S4, S15) and two 46,XY CAIS labia majora fibroblasts strains (ARD411, ARD1097) were selected (Table 2) for genome-wide analysis by microarray and subsequent qRT-PCR.

Cell culture treatment chart

No	Treatment type
1	10 nM DHT (in ethanol) plus 2 µg/ml AZA (in acetic acid)
2	10 nM DHT (in ethanol) plus acetic acid
3	ethanol plus AZA (in acetic acid)
4	ethanol plus acetic acid
5	10 nM DHT (in ethanol)
6	Equal concentration of ethanol as in treatment type 5

The cells were treated with either (1) 10 nM DHT (in ethanol) plus 2 µg/ml AZA (in acetic acid); (2) 10 nM DHT (in ethanol) plus acetic acid; (3) ethanol plus AZA (in acetic acid); and (4) ethanol plus acetic acid. DHT (Merck, Darmstadt, Germany) was dissolved in ethanol (final dilution in culture media = 1:10,000), AZA (Sigma, Taufkirchen, Germany) was dissolved in acetic acid (final dilution in culture media = 0.5%). Passage numbers were between 3 and 9 and 1 x 10^6 cells were seeded per culture (T-175 flasks) (day 1). Hormone stimulations started 24 hrs after seeding the cells (day 2) and were repeated once every 48 hrs for a total of 6 times (last treatment at day 14). After the final hormone stimulation, cells remained in culture for 96 hrs prior to harvesting (day 18). By this method, fibroblasts were confluent leading to stable gene transcription profiles on microarrays (Holterhus *et al*. 2003). In the second independent experimental set of fibroblast cultures (phenotypic normal males: S3, S4, S5, S8, S9, S12, S13, S15, CAIS: ARD682, ARD1144, ARD402, PAIS: ARD446,

ARD534, ARD001, ARD084, ARD377, ARD659, 17ß-HSDIII deficiency: ARD111, ARD1373) cells received either 10 nM DHT plus ethanol or only the solvent ethanol under the same treatment regimen as described above and RNA was subsequently subjected to qRT-PCR.

2.3.4 Isolation of Peripheral blood mononuclear cells (PBMCs)

Peripheral blood from apparently healthy volunteers was obtained at 10-10:30 A.M and 1-1:30 P.M. The Donors age ranges from 24-40.

1) A total volume of 10 ml blood were used for isolating PBMCs. Since the blood was not mixed with EDTA it was processed immediately after obtaining from the donor.
2) The blood was transferred into 50 ml of falcon tube and washed with 1x Dulbecco's PBS (PAA, Pasching, Austria) and adjusted to a final volume of 35 ml.
3) A total volume of 15 ml Ficoll reagent at 4°C (Biochrom AG) was transferred into a 50 ml falcon tube.
4) The mixture of blood and PBS (35 ml) was overlaid slowly and carefully on the top of Ficoll without mixing.
5) The falcon tubes were centrifuged at 2,400 rpm for 20 min.
6) The cell ring (containing PBMCs) was obtained with the help of a 10 ml pipette and transferred into 15 ml falcon tube and centrifuged at 2,000 rpm for 10 min.
7) The supernatant was discarded and the cell pellet was washed carefully with 10 ml of 1x PBS and centrifuged at 2,000 rpm for 10 min.
8) The supernatant was discarded and the pellet was washed one more time with 10 ml of 1x PBS. 10 µl of cells were used for counting with the hemocytometer. A volume of 10 µl PBMCs were transferred into 90 µl of Türks solution (1:10 dilution) present in an Eppendorf tube. Cell counting was performed in a similar way as mentioned for fibroblasts.

2.3.5 Culturing and treating of PBMCs with dihydrotestosterone (DHT)/ Ethanol (EtOH)

PBMCs were (1 x 10^6 cells/ vial) incubated in an orbital shaker for 24 hrs with 200 rpm at 37°C and 5% CO_2. Experimental PBMCs were treated with 50 nM DHT in EX VIVO-15 medium (Cambrex Bio Science Inc, Walkersville, MD, USA) for 24 hrs and control PBMCs were treated with equal concentration of ethanol for 24 hrs. RNA was isolated after 24 hrs of treatment.

2.4 Molecular biological techniques

2.4.1 Isolation of total RNA

Total RNA isolation from fibroblasts and PBMCs was performed using Trizol treatment (Invitrogen, Paisley, United Kingdom) followed by RNeasy mini kit protocol (Qiagen, Hilden, Germany).

1) The fibroblasts were harvested by scraping with 5 ml of 1x Dulbecco's PBS at 4°C. The cells were centrifuged at 1000 rpm, 6°C for 15 min.
2) The supernatant was discarded and the cells were washed with 5 ml of 1x Dulbecco's PBS and then centrifuged them at 2000 rpm at 6°C for 15 min. The supernatant was discarded.
3) 1 ml of Trizol was added to the cell pellet (1 x 10^7 cells). The cell pellet was thoroughly mixed by vortexing and pipetting up and down. The cell suspension was incubated for 30 min at room temperature. The cell suspension was transferred to 1.5 ml Eppendorf tube.
4) 200 µl of chloroform (J.T. Becker) were added to the cell suspension and mixed well for every 30 seconds and incubated for 2 min at room temperature.
5) The Eppendorf tubes were centrifuged at 12,000 rpm for 15 min at 6°C.
6) The upper aqueous phase was collected (approximately 700 µl) into a new Eppendorf tube without disturbing the protein or phenol layer.
7) An equal amount of 70% ethanol was added to the upper phase and mixed with a pipette for 2-3 times.
8) 700 µl of the above mixture was applied to the RNeasy column and centrifuged for 10,000 rpm for 15 seconds.

9) The flow-throw was discarded and again 700 µl of the ethanol-cell suspension (step 7) were applied to the RNeasy column.
10) The RNeasy column was centrifuged at 10,000 rpm for 15 seconds. The flow-throw was discarded.
11) To the RNeasy columns 700 µl of buffer RW1 were applied and centrifuged at 10,000 rpm for 15 seconds.
12) The column was transferred to a new collection tube. 500 µl of RPE buffer (containing ethanol) were applied to the RNeasy columns and centrifuged at 10,000 rpm for 15 seconds. The flow-throw was discarded.
13) Again 500 µl buffer RPE were applied to the RNeasy column and centrifuged at 10,000 rpm for 2 min.
14) The RNeasy column was transferred to a new 1.5 ml Eppendorf tube. 50 µl of RNase free water were applied to the center of the membrane and centrifuged at 10,000 rpm for 1 min.
15) The resulting elute was applied one more time to the centre of the column and centrifuged for 10,000 rpm for 1 min.
16) The DNA contamination was removed by using DNA free kit (Ambion, Austin, USA).
17) 5 µl of 10x DNase buffer and 1 µl DNase I were applied to the eluted RNA mixed well by vortexing and incubated for 20 min at 37°C.
18) 5 µl of DNase-inactivation solution was applied to the above mixture, mixed well by vortexing.
19) This mixture was incubated for 2 min at room temperature (mixed 1 time during the incubation) and centrifuged at 10,000 rpm for 1 min.
20) RNA solution was transferred to a new Eppendorf tube and stored at -80°C. RNA quantity and quality were analyzed with 1:1 ratio of RNA and water by using 2100 Bioanalyzer (Agilent, Palo Alto, USA) and RNA 6000 Nano assay (Agilent, Palo Alto, USA).

2.4.2 RNA (Total RNA, antisense RNA) quantification analysis by using 2100 Bioanalyzer

1) All the reagents were equilibrated to the room temperature for 30 min before use.
2) A total of 550 µl of RNA 6000 Nano gel matrix was filtered by using a spin filter column in a microcentrifuge and spinned at 4000 rpm for 10 min.
3) The filtered gel was aliquoted and used within one month of preparation.
4) 1 µl of RNA 6000 Nano dye was added to 65 µl of filtered Nano gel, mixed thoroughly by vortexing and centrifuged at 14000 rpm for 10 min.
5) RNA Nano chip was taken on to the chip priming station. 9 µl of the above gel mix was taken onto the well of a RNA Nano chip marked as G. The plunger was set to 1 ml, the priming station was closed and the plunger was pressed until it locked.
6) After 30 seconds the plunger was released and allowed it until it reaches it is own position at 1 ml.
7) 9 µl of gel was added to each gel marked as G.
8) 5 µl of the RNA 6000 Nano Marker was taken onto the well marked with the ladder symbol and each of the 12 sample wells.
9) 1 µl of RNA 6000 ladder (Ambion Inc), experimental samples (1:1 RNA, water) were denatured at 70°C for 2 min and cooled down to 4°C.
10) 1 µl ladder or experimental samples were loaded onto the wells. The chip was finally taken onto the IKA vortexer (Agilent) and mixed at 2,400 rpm for 1 min.
11) The chip was taken onto the Agilent 2100 bioanalyzer and measured the RNA quality and quantity.

2.4.3 RNA amplification for cDNA and Oligo microarrays

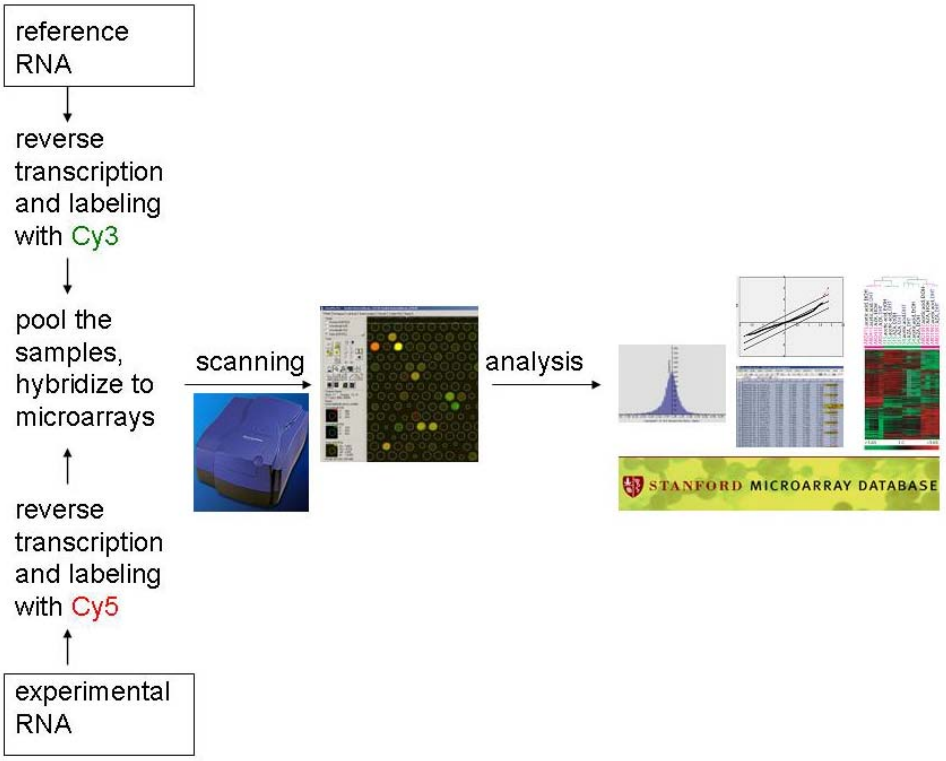

Figure 10. Schematic representation of microarray procedure and data analysis in the Stanford microarray database (SMD)

For cDNA microarrays RNA was amplified by using MesageAmp^{TM} II aRNA kit (Ambion, Huntigdon, UK), for oligo microarrays Amino Allyl MessageAmp™ II with Cy™3/Cy™5 (1:3) (Ambion, Huntigdon, UK) kit was used. The universal human reference RNA (Stratagene Europe, Amsterdam, The Netherlands) was amplified in a single batch by using the above kits with similar conditions.

The procedure of RNA amplification until *in vitro* transcription was the same.

Reverse transcription for synthesizing the First strand cDNA

1) The initial RNA input was approximately 400 ng to 1000 ng not exceeding the volume of 11 µl. 1 µl of T7 Oligo (dt) primer was added to the RNA probe. The final volume was adjusted to 12 µl with nuclease-free water.

2) The above mixture was stirred briefly by vortexing and incubated for 10 min at 70°C in a thermal cycler. After the incubation the reaction tubes were centrifuged briefly (~5 sec) and placed on ice for 2 min.
3) 8 µl of Reverse Transcription master mix was added to the above mixture and mixed thoroughly by pipetting up and down for 2-3 times, followed by quick spin.

First strand master mix (Reverse transcription mixture)

Amount	Component
2 µl	10x First Strand buffer
4 µl	dNTP mix
1 µl	RNase inhibitor
1 µl	ArrayScript

4) The above mixture was incubated at 42°C in a thermal cycler for 2 hrs. After 2 hrs the mixture was centrifuged briefly and placed on ice.

Second strand cDNA synthesis

5) At the end of the 2 hrs second strand cDNA synthesis master mix was prepared

Second strand master mix

Amount	Component
63 µl	Nuclease free water
10 µl	10x second strand buffer
4 µl	dNTP mix
2 µl	DNA polymerase
1 µl	RNase H

5) The above mixture was stirred gently by vortexing and centrifuged briefly. Later on 80 µl of this mixture were added to each sample (first strand mixture), mixed thoroughly by pipetting up and down for 2-3 times and centrifuged briefly.
6) The above samples were incubated at presetted thermal cycler at 16°C for 2 hrs. The lid of the thermal cycler was left open due to the possible varying temperatures of thermal block and lid.
7) At the end of the 2 hrs the reactions were placed on ice and immediately proceeded to cDNA purification.

cDNA purification

9) 250 µl of cDNA binding buffer were added to the above mixture and mixed thoroughly by pipetting up and down for 2-3 times.
10) The above mixture was loaded onto the middle of the mini-column of the cDNA filter cartridge and centrifuged at 10,000 rpm for 1 min.
11) The flow-throw was discarded and the mini-column was transferred to cDNA wash tube.
12) 500 µl of cDNA wash buffer (including ethanol) were added to the cDNA filter cartridge and centrifuged at 10,000 rpm for 1 min.
13) The flow-throw was discarded and the cDNA filter cartridge was transferred to a cDNA elution tube.
14) 10 µl of preheated (52°C) nuclease free water were added to the center of the filter in the cDNA filter cartridge, incubated for 2 min and centrifuged at 10,000 rpm for 1.5 min.
15) Again 10 µl of preheated (52°C) nuclease free water were pipetted into to the center of the filter in the cDNA filter cartridge, incubated for 2 min and centrifuged at 10,000 rpm for 1.5 min.
16) The resulting cDNA eluate (~ 16 µl) was proceeded immediately to *in vitro* transcription.

In Vitro Transcription (IVT) to synthesize antisense RNA(aRNA)

IVT master mix for *cDNA arrays* IVT master mix for *Oligo arrays*

Amount	**Component**	**Amount**	**Component**
4 µl	T7 ATP solution (75 mM)	3 µl	Amino-allyl UTP (50 mM)
4 µl	T7 CTP solution (75 mM)	12 µl	ATP, CTP, GTP mix (25 mM)
4 µl	T7 GTP solution (75 mM)	3 µl	UTP (50 mM)
4 µl	T7 UTP solution (75 mM)	4 µl	T7 10x Reaction buffer
4 µl	T7 10x reaction buffer	4 µl	T7 Enzyme mix
4 µl	T7 enzyme mix		

17) The above mixture was mixed by gentle vortexing and centrifuged briefly. 24 µl of the IVT mixture for cDNA arrays and 26 µl for Oligo arrays were added to each experimental sample sample (~16 µl), thoroughly mixed by pipetting up and down for 2-3 times followed by quick spin.
18) The IVT reaction was performed in a hybridization oven at 37°C for 14 hrs. After the incubation the reaction was stopped by adding 60 µl of nuclease free water.

aRNA purification (same procedure for RNA amplified for cDNA microarrays and Oligo microarrays)

350 µl of aRNA binding buffer were added to each aRNA sample:
19) 250 µl of ACS grade ethanol (100%) were applied to each aRNA sample, mixed by pipetting up and down for 3 times. The aRNA samples were neither mixed by vortexing nor centrifuged. The samples were processed immediately to the next step.
20) Samples were loaded onto the center of the aRNA filter cartridge and centrifuged at 10,000 rpm for 1 min. The flow-through was discarded

21) 650 µl of wash buffer were added to the aRNA filter cartridge and centrifuged at 10,000 rpm for 1 min. The flow-through was discarded and the filter cartridge was centrifuged one more time for 1 min at 10,000 rpm to minimize the carry over of ethanol. The aRNA filter cartridge was transferred to a new collection tube.
22) 100 µl of preheated (58°C) nuclease free water were added to the aRNA filter cartridge, incubated at room temperature for 2 min and centrifuged at 10,000 rpm for 1.5 min.
23) 1 µl of aRNA was aliquoted for quality and quantity analysis and rest of the aRNA was stored at -80°C.

2. 5 Hybridization of cDNA microarrays (Fibroblast project)

Spotted cDNA microarrays (Stanford Functional Genomics Facility, Stanford, CA, USA) were used for gene expression studies containing approximately 43,000 features representing 32,968 unique human genes.

2.5.1 Post processing of cDNA arrays

Using a diamond scribe, the non-printed side of the 4 corners of the array was denoted.

Array stabilization and immobilization

1) The arrays were rehydrated by holding slides over a bath containing 1x SSC (Flucka, Switzerland) at room temperature for 3 min.
2) The arrays were snap-dried (DNA side up) on a 99°C hot plate (Techne Dri-Block DB-1) for 3 sec until the vapor disappears.
3) The DNA on the arrays were cross-linked with UV light (UV cross linker, Amershan Life Sciences, USA) at 65 mJoules (650 x 100uJ).

2.5.2 Pre Hybridization

1) Pre hybridization buffer was pre-warmed in a coplin jar at 42°C waterbath for 60 min.

Composition of pre hybridization buffer

Amount	Component	Final concentration
300 ml	Millipore water	
100 ml	filtered 20x SSC	5x
4 ml	BSA (10 mg/ ml stock solution)	0.1 mg/ml
4 ml	SDS (10% stock solution)	0.1%

2) The arrays were transferred to a slide rack and placed onto the pre-hybridization buffer and incubated at 42°C for 60 min.
3) The arrays were rinsed in 0.1x SSC for 5 min at room temperature. This step is repeated again in a new 0.1x SSC solution for 5 min.
4) The arrays were transferred to Milli-Q water (Millipore) and incubated for 30 sec at room temperature.
5) The arrays were spin dried in a centrifuge (Beckman Coulter Allegra^{TM} X-22R) at 1000 rpm for 10 min. The arrays were used on the same day (possibly within an hour).

2.5.3 Labeling of aRNA

Preparation of master mix 1

Component	Sample	Control
RNA	3 µg	2 µg
Random hexa primer	2 µl	2 µl
Nuclease free water	adjusted to 15.4 µl	adjusted to 15.4 µl
Total	14.6 µl	14.6 µl

1) The above sample and reference RNA were incubated at 65°C for 10 min and cooled down to 4°C in a thermal cycler.

Preparation of master mix 2

Component	**Sample**	**Control**
5x 1st strand buffer	6 µl	6 µl
0.1 M DTT	3 µl	3 µl
dNTPs	0.6 µl	0.6 µl
Cy3	----	3 µl
Cy5	3 µl	----
SuperScript II	2 µl	2 µl
Total	14.6 µl	14.6 µl

2) The above RNA samples were mixed by brief vortexing and then centrifuged. Care was taken not to have direct sun light and maintained the possible dark conditions to protect the Cy dyes.
3) Mix 1 (RNA labeled with Random hexa primers) were transferred to master mix 2 (step 2) and incubated for 60 min at 42°C in a thermal cycler.
4) After 60 min 1 µl of SuperScript II was added to each RNA sample and incubated again for 60 min at 42°C.
5) The reaction was stopped by adding 15 µl of 0.1 N NaOH (J.T. Becker), 2 mM EDTA (Flucka). The mixture was stirred briefly by vortexing.
6) The above reaction mixture was neutralized by adding 15 µl of 0.1 N HCl (J.T. Becker).
7) 380 µl of TE (Flucka) were added to Microcon YM-30 column (Microcon).
8) To the microcon columns 60 µl of test RNA and 60 µl of reference RNA probes were added.

9) The microcon columns were centrifuged at 12,000 rpm for 8 min. After the centrifugation the possible breaking of membranes were eye inspected.
10) The flow-through was discarded and 450 µl of TE buffer were added to the microcon columns and centrifuged at 12,000 rpm for 8 min.
11) The flow-through was discarded and 474 µl of the below master mix were added to clean the labeled RNA.

Amount	Component
450 µl	TE buffer
20 µl	1 µg/ µl of human Cot-1 DNA
2 µl	10 µg/ µl of Poly A RNA
2 µl	10 µg/ µl tRNA

12) The microcon columns were centrifuged at 12,000 rpm for 9 min. At the end the flow-through was discarded.
13) The columns were centrifuged few times (to concentrate the labeled RNA) at 12,000 rpm for 1 min until the labeled mix reaches the volume of 28 µl.
14) The microcon column was kept upside down in a new collection tube and centrifuged at 14,000 rpm for 14 sec.
15) The probe volume was adjusted to 32 µl with 1x TE buffer.
16) To the probe 6.75 µl of 20x SSC and 1.2 µl of 10% SDS were added. Care was taken not to introduce excess SDS or bubbles.
17) The samples were denatured at 99°C for 2 min in a heating block and centrifuged at 14,000 rpm for 30 min to precipitate the unbound labeling material.
18) 15 µl of 3x SSC were added to the well of hybridization chamber to maintain the humidity.
19) The prehybridized array was taken onto the hybridization chamber.
20) Approximately 40 µl of probe were loaded onto the middle of the microarray. Care was taken not to introduce any bubbles.

21) With the help of a forceps a coverslip (Erie scientific Co, Ports) was placed on the top of the microarray without introducing any bubbles.
22) On the top of the 4 corners of the coverslip 3 µl of 3x SSC buffer were loaded.
23) The hybridization chamber was sealed carefully with bolts and placed on a preheated water bath at 65°C. The arrays were incubated for 16 hrs.

2.5.4 Preparation of Washing buffers and washing of Arrays

Wash	Description	Volume (ml)	SSC	SDS (10%) (ml)
1A	2x SSC, 0.03% SDS	500	50 ml of 20x SSC	1.5
1B	2x SSC, 0.03% SDS	500	50 ml of 20x SSC	1.5
1C	2x SSC	500	50 ml of 20x SSC	
2	1x SSC	500	25 ml of 20x SSC	
3	0.2x SSC	500	5 ml of 20x SSC	
4	0.1x SSC	500	2.5 ml of 20x SSC	

24) Wash 1A: . Each array was kept in this chamber until cover slip ditaches the array.
25) Wash 1B: The arrays were rinsed until 2 min at room temperature.
26) Wash 1C: The arrays were rinsed until 2 min at room temperature.
27) Wash 2: The arrays were rinsed until 2 min at room temperature.
28) Wash 3: The arrays were rinsed until 2 min at room temperature.
29) Wash 4: The arrays were rinsed until 2 min at room temperature.
30) At the end of the washing, arrays were taken to the centrifuge along with the washing chamber.
31) The arrays were centrifuged at 1000 rpm for 10 min.
32) The dried arrays were transferred to a dry array case and scanned with Genepix 4000B microarray scanner (Axon, Foster City, USA).

33) Images were analyzed with GenePix Pro 4.0 software (Axon Instruments, Foster City, USA).

2.6 Hybridization of Oligo microarrays (Blood project)

The oligonucleotide microarrays (Stanford Functional Genomics Facility, California, USA) consists of 44,544 70mer probes (approximately 40,570 exonic probes) that were designed by using a transcriptome-based annotation of exonic structure for genomic loci.

1) The aRNA was quantified and 5 µg of treated sample and 3.5 µg of amino allyl universal human reference RNA were vaccum dried in a separate PCR stips by using a speedvac (Christ RVC 2-18) until complete dry.
2) To the dried RNA samples 9 µl of coupling buffer were added and re-suspended thoroughly by gentle vortexing.
3) 11 µl of dimethyl sulfoxide (DMSO, Ambion) were added to each tube of Cy3 and Cy5, mixed gently by vortexing. Cy3 or Cy5 tubes were light protected and used within a month of preparation and stored at -20°C.
4) 8.25 µl of Cy5 were added to the experimental RNA and 3.25 µl of Cy3 were added to the common references RNA. These individual reaction tubes were incubated at room temperature in dark (in a closed drawer) for 30 min.
5) To quench the reaction 4.5 µl of Hydroxlyamine were added to each reaction tube and mixed well by gentle vortexing and incubated at room temperature in a dark place for 15 min.
6) 5.5 µl of nuclease-free water were added to each sample to bring the volume upto 30 µl.

2.6.1 Purification of labeled aRNA

7) To each sample 105 µl of aRNA binding buffer were added.
8) 75 µl of ACS grade 95% ethanol were added to each sample and mixed well by pipetting up and down for 3 times. Vortexing and centrifuging were avoided.
9) The entire sample was applied to labeled aRNA filter cartridge.
10) The samples were centrifuged at 10,000 rpm for 1 min. The flow-through was discarded.

11) To each filter cartridge 500 µl of aRNA wash buffer were applied and centrifuged at 10,000 rpm for 1 min. The flow-through was discarded and the filter cartridge was centrifuged again at 10,000 rpm for 1 min to remove the excess or carry over of ethanol.
12) The labeled aRNA filter cartridge was taken onto a new elution tube. To the center of the labeled aRNA filter cartridge 10 µl of preheated water (55°C) were applied and incubated for 2 min at room temperature.
13) The filter cartridge was centrifuged at 10,000 rpm for 1.5 min.
14) Again 10 µl of preheated water (55°C) were applied and incubated for 2 min at room temperature.
15) The filter cartridge was centrifuged at 10,000 rpm for 1.5 min.
16) The resulting labeled aRNA was used for microarray hybridization.
17) The experimental samples (treated or control RNA-labeled with Cy5), control samples (universal human reference RNA labeled with Cy3) were pooled and the final volume was adjusted to 20 µl with the help of a nuclease-free water.
18) To the pooled RNA 3 µl of 10x Fragmentation buffer (Ambion) were applied and incubated for 15 min at 70°C in a thermal cycler.
19) The reaction was stopped by adding 3 µl of Stop solution (200 mM EDTA, pH 8.0) .

Preparation of probe for hybridization

Amount	**Reagents**
2 µl	10 µg/µl Cot-1 human DNA
2 µl	10 µg/µl polyA RNA
2 µl	10 µg/µl tRNA
Total volume	6 µl

20) The above master mix was applied to the pooled-labeled RNA probe, then 5.95 µl of 20x SSC were added. To this 0.84 µl of 1 M (pH 7.0) HEPES buffer followed by 1.05 µl of 10% SDS were added.
21) The above probe was denatured at 99°C for 2 min in a thermal cycler.
22) The probe was centrifuged at 13,000 rpm for 30 min.

23) 15 µl of 3x SSC were added to the well of hybridization chamber to maintain the humidity.
24) The pre-hybridized array was taken onto the hybridization chamber.
25) Approximately 40 µl of probe were loaded onto the middle of the microarray. Care was taken not to introduce any bubbles.
26) With the help of a forceps a coverslip was placed on the top of the microarray without introducing any bubbles.
27) On the top of the 4 corners of the coverslip 3 µl of 3x SSC buffer were loaded.
28) The hybridization chamber was sealed carefully with bolts and placed in a preheated water bath at 65°C. The arrays were incubated for 16 hrs.

2.6.2 Washing of Arrays

Preparation of washing buffers

Wash	Description	Volume (ml)	SSC	SDS (10%) (ml)
1A	2x SSC, 0.03% SDS	500	50 ml of 20x SSC	1.5
1B	2x SSC, 0.03% SDS	500	50 ml of 20x SSC	1.5
1C	2x SSC	500	50 ml of 20x SSC	
2	1x SSC	500	25 ml of 20x SSC	
3	0.2x SSC	500	5 ml of 20x SSC	

29) Wash 1A (pre warmed at 60°C): Each array was kept in this chamber until cover slip detaches the array.
30) Wash 1B (pre warmed at 60°C): The arrays were rinsed until 2 min at room temperature.
31) Wash 1C: The arrays were rinsed until 2 min at room temperature.
32) Wash 2: The arrays were rinsed until 2 min at room temperature.
33) Wash 3: The arrays were rinsed until 2 min at room temperature.
34) Wash 4: The arrays were rinsed until 2 min at room temperature.

35) At the end of the washing the arrays were taken to the centrifuge along with the washing chamber.
36) The arrays were centrifuged at 1000 rpm for 10 min.
37) The dried arrays were transferred to a dry array case and scanned with Genepix 4000B microarray scanner (Axon, Foster City, USA).
38) Images were analyzed with GenePix Pro 4.0 software (Axon Instruments, Foster City, USA).

2.7 Microarray data analysis for Fibroblast project

Only spots with fluorescence signals of $\geq$ 1.5-fold over array background in either the experimental (Cy5) or the reference (Cy3) channel were considered based on the 16 microarray experiments representing the four experimental conditions corresponding to the four used cell strains in the first set of treatments. Cy5/Cy3 fluorescence ratios for all genes were normalized to obtain an average absolute $\log_2$ red / green ratio of 0 in each microarray. Fluorescence ratios for each gene were centered (by mean) and genes with at least 80% interpretable data in the microarrays were used. We further considered only those transcripts whose $\log_2$ red (Cy5) / green (Cy3) ratio differed from the mean expression level across all 16 experiments by at least 1.0 (corresponding to 2-fold up - or 2-fold down-regulation) in at least four of 16 experiments. Data were displayed by TreeView (Eisen *et al*. 1998). We subsequently performed unsupervised hierarchical clustering analysis (Pearson correlation metrics, average linkage clustering) (Eisen *et al*. 1998). In addition, we applied the Significance Analysis of Microarrays (SAM) procedure (Tusher *et al*. 2001) to identify transcripts with statistically significant differences in expression levels on microarrays comparing all DHT-treated samples with all untreated samples in the normal scrotum fibroblasts independent from the presence or absence of AZA. Moreover, we have compared all AZA-treated samples with all non-AZA samples independent from DHT treatment or AR-status by SAM.

2.8 Microarray data analysis for Blood project

Only spots with fluorescence signals of ≥ 1.5-fold over array background in either the experimental (Cy5) or the reference (Cy3) channel were considered based on the 12 microarray experiments representing the four experimental conditions corresponding to the four used cell strains in the first set of treatments. Cy5/Cy3 fluorescence ratios for all genes were normalized to obtain an average absolute $\log_2$ red / green ratio of 0 in each microarray. Fluorescence ratios for each gene were centered (by mean) and genes with at least 80% interpretable data in the microarrays were used. We further considered only those transcripts whose $\log_2$ red (Cy5) / green (Cy3) ratio differed from the mean expression level across all 12 experiments by at least 1.0 (corresponding to 2-fold up - or 2-fold down-regulation) in at least five of 12 experiments. Data were displayed by TreeView (Eisen *et al*. 1998). We subsequently performed unsupervised hierarchical clustering analysis (Pearson correlation metrics, average linkage clustering) (Eisen *et al*. 1998).

2.9 Total RNA amplification, quantitative real time PCR (qRT-PCR) of APOD

Total RNA was amplified according to the manufacturer's instructions using the First strand cDNA synthesis kit (Fermentas, Hannover, Germany).

2.9.1 RNA amplification for qRT-PCR

1) 1000 ng of total RNA was taken onto 500 µl Eppendorf tube.
2) 2 µl of random hexamer were added to the total RNA and the final volume was adjusted to 26 µl with nuclease free water. This mixture was mixed gently and centrifuged briefly.
3) The RNA samples were incubated at 70°C for 5 min.

Preparation of master mix

Amount	Reagents
8 µl	5x Reverse transcription buffer
1 µl	RNase inhibitor
4 µl	dNTP

4) 13 µl of the above master mix was added to each RNA sample and incubated at 25°C for 5 min.
5) 1 µl of Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase was added to the RNA samples and incubated at 42°C for 1 hr.
6) The samples were denatured at 70°C for 10 min and cooled down to 4°C.

2.9.2 Procedure of qRT-PCR for APOD

QRT-PCR was applied to a total of 8 scrotum fibroblasts strains, 5 CAIS labia majora fibroblasts strains, 6 PAIS fibroblasts strains and 2 17ß-HSDIII fibroblast strains (Table 2). QRT-PCR was performed using the QuantiTect SYBR Green PCR kit (QIAGEN, Hilden, Germany). The primers used for PCR (TIB MOLBIOL, Berlin, Germany) had the following sequences: APOD forward primer 5'-CCACCCCAgTTAACCTCACA–3', and APOD reverse primer 5'-gTgCCgATggCATAAACC-3'. Succinate Dehydrogenase Subunit A (SDHA) was used as housekeeping gene for normalization. SDHA forward primer was 5'-ACCAggTCACACACTgTTgC-3' and SDHA reverse primer was 5'-CgTAgAAATgCCACCTCCA-3'. PCR was carried out on the LightCycler (Roche Diagnostics, Mannheim, Germany). In total 18 µl of PCR master mix including primers and 2 µl of cDNA (6.25 ng/µl) were pipetted into each capillary. The PCR conditions were 95°C for 15 min, 45 cycles of 94°C for 15 sec, 52°C for 25 sec and 72°C for 25 sec. Standard curves for APOD and SDHA were obtained by running the PCR with different dilutions of reference cDNA. Standard curves were used to assess the efficiency correction for the experimental probes. Melting curves were analyzed to make sure that the amplified product was specific for APOD, SDHA and devoid of primer dimers.

3. Results

3.1 DHT mediated gene expression patterns in GSF

Filtering of all genes of all the 16 microarrays (experimental set 1) as described above resulted in 3,601 transcripts. Unsupervised hierarchical clustering of these transcripts across the 16 microarray experiments demonstrated distinct, reproducible patterns of gene expression (Figure 11).

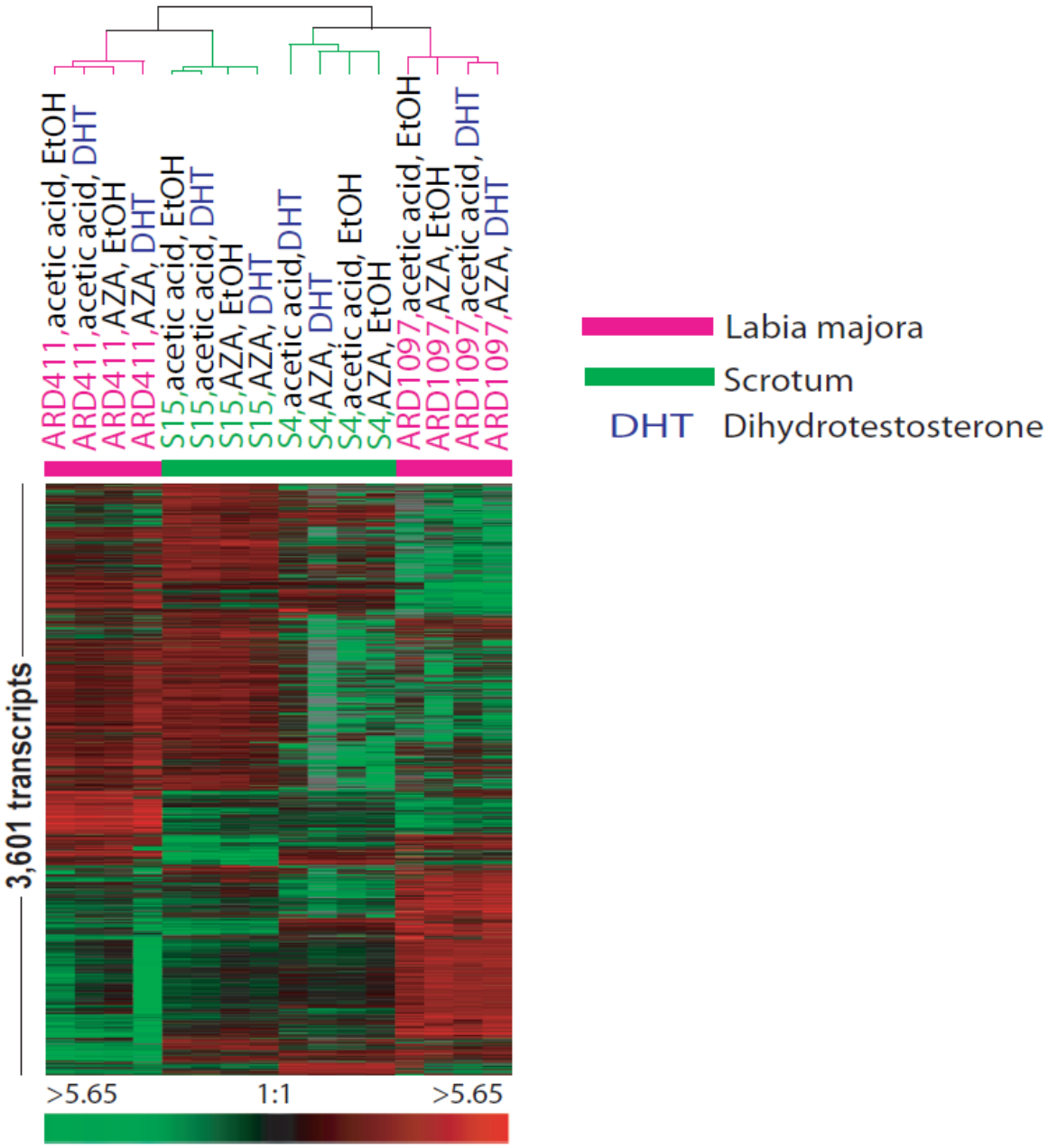

Figure 11. Unsupervised hierarchical clustering analysis of scrotum and 46 XY labia majora fibroblasts (experimental set 1). The heatmap represents hierarchical clustering of

3,601 transcripts which had at least 80% interpretable data across the 16 microarrays and whose expression levels were at least 2-fold different from the mean expression level across all samples in at least 4 experiments. Transcripts are represented in rows and experiments in columns. Expression values per gene are centered by the mean $\log_2$ Cy5/Cy3 normalized ratio. Increasing red intensity corresponds to higher relative transcript levels compared to the mean expression level across all experiments. Increasing green intensity corresponds to relatively decreased transcript levels compared to the mean. The cluster dendrogram demonstrates the degree of relatedness (Pearson correlation) between the expression patterns of the 3,601 genes in the 16 fibroblast samples. The length of the arms of the dendogram reflects the degree of correlation between the samples.

Clustering sorted all microarray experiments according to the cell strain indicating the existence of highly strain specific, stable baseline gene transcription signatures independent from the treatment of the cells. Therefore, strain identity was the overriding biological pattern causing gene expression differences in the 16 microarrays dataset. From the methodological perspective, this observation confirms the high reproducibility of our cell culture strategy in combination with the microarray procedures (Figure 11) as also previously published (Holterhus PM *et al*. 2007). However, our unsupervised analysis did neither expose obvious influences of DHT treatment nor of AZA-treatment suggesting that the cells responded only little to either of these two treatments. Therefore, at this stage of analyses, our finding on scrotal fibroblasts seemed to agree with our previous work showing a lack of response of foreskin fibroblasts to many different protocols of DHT treatment (Holterhus PM *et al*. 2003, Bebermeier JH *et al*. 2006).

Since unsupervised analysis did not show obvious DHT-effects, we performed a supervised statistical search for significant DHT-regulated genes using the well-established Significance Analysis of Microarrays (SAM) procedure (Tusher VG *et al*. 2001). Using this algorhythm, we compared all DHT-treated normal scrotum fibroblast experiments with all non-DHT-treated normal scrotum fibroblast experiments, independent of the presence or absence of AZA. Based on the analysis of all expressed genes with 80% interpretable data in the fibroblasts (23,863 genes), SAM identified three transcripts, namely APOD (Accession number AA456975), Phosphoglucomutase 5 pseudogene 1 (PGM5P1, accession number AA598500) and the transcribed locus AA459734 using a SAM filter with a false discovery rate of 0% (Figure 12a). Figure 12b compiles the gene expression data of the above three transcripts in the 16 microarray experiments. In addition to the obvious strain-specific

variability of baseline expression of all three transcripts (e.g., see ARD1097 versus ARD411), Figure 12b illustrates the striking response in the two scrotum strains to DHT treatment but no response in the two CAIS strains. SAM analysis did not reveal reproducible changes in gene expression in response to treatment with AZA.

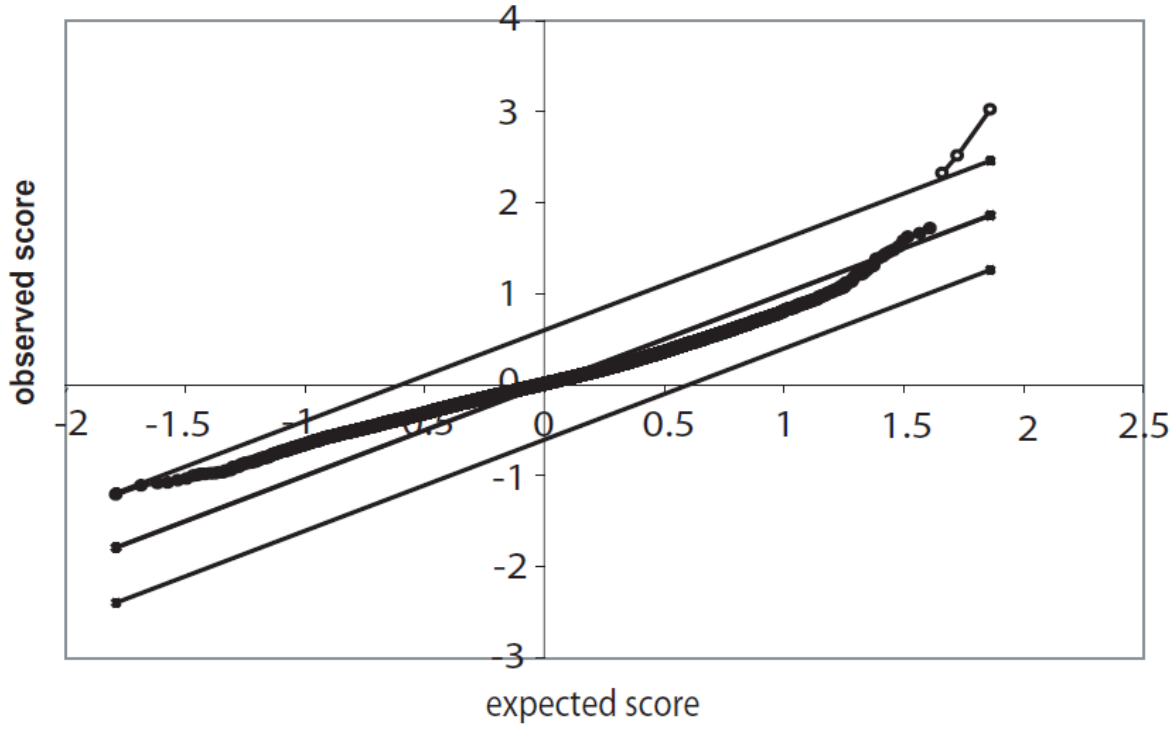

Figure 12a. Identification of androgen induced transcripts by significance analysis of microarrays (SAM)

Plot of observed scores (Y-axis) versus expected scores (X-axis) according to (Tusher VG *et al*. 2001) of DHT-induced transcripts calculated by SAM-analysis on the basis of 16 microarrays in experimental set 1. The open dots represent the transcripts which are significantly induced by DHT at a false positive discovery rate of 0%.

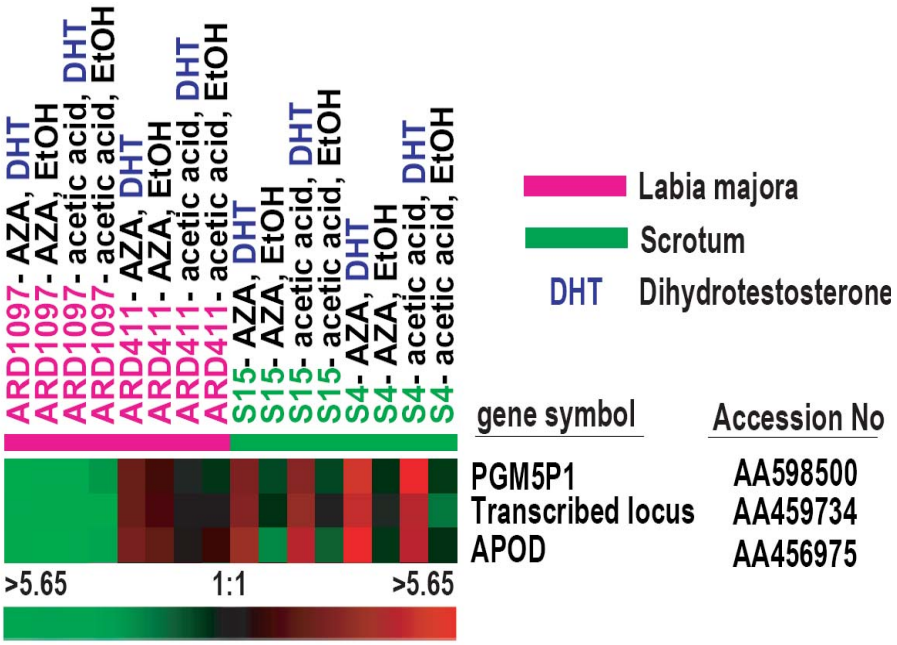

Figure 12b. Color-coded display of expression values of DHT- induced transcripts (experimental set 1)

The rows in the heatmap show the 3 transcripts APOD, PGM5P1 and the transcribed locus AA459734 which were identified to be significantly induced by DHT as measured on microarrays using SAM. Expression values for each transcript were centered by mean. Red – green color coding has the same meaning as in Figure 11.

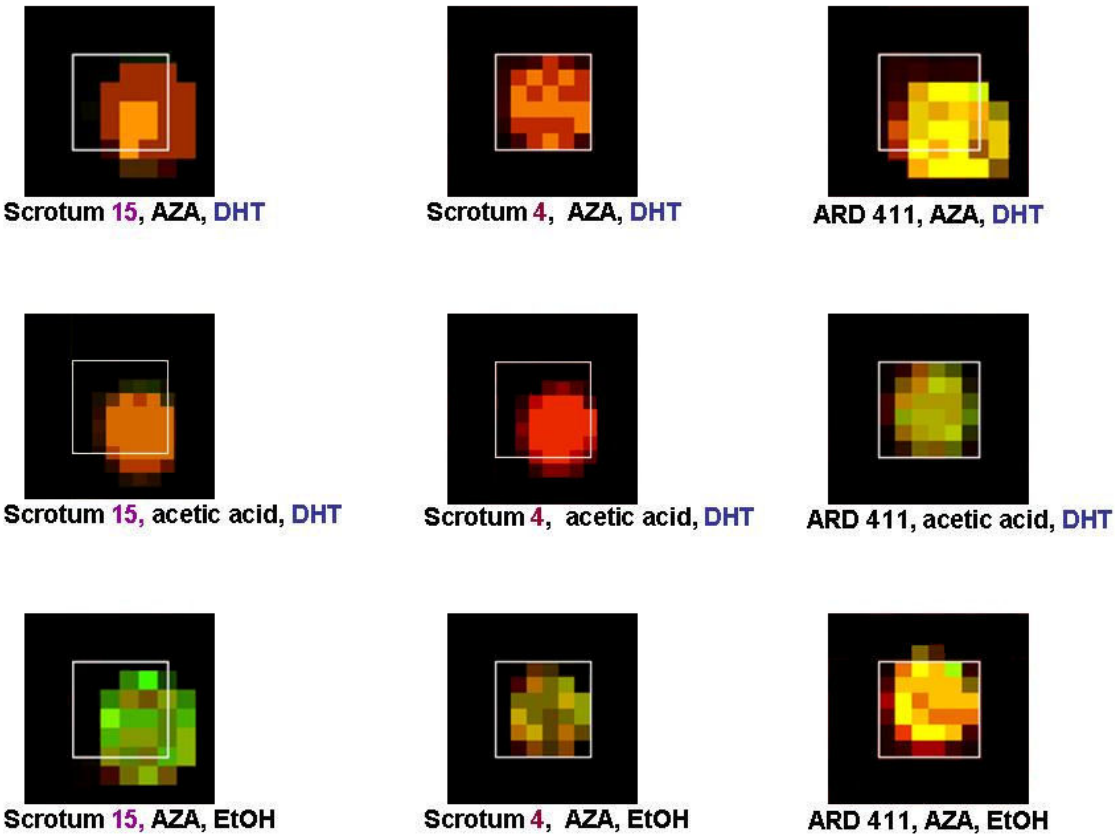

Figure 13. APOD gene expression images in two different scrotum and one labia majora cells in different treatment conditions (Type-1 microarrays, not discussed).

The Same RNA was used for type-1 and type-2 microarrays. APOD induction was observed in scrotum 15 and 4 treated with combination of AZA and DHT as well as acetic acid and DHT. In contrast, ARD 411 treated with combinations of AZA and DHT as well as acetic acid and DHT. APOD expression was limited to baseline in case of scrotum 15 and 4 as well as ARD 411 in the absence of DHT. This supports the specificity of DHT mediated induction of APOD in scrotum cells.

3.2 Confirmation of APOD expression values of microarrays by using qRT-PCR

We subsequently selected APOD for our validation experiments. To directly confirm the gene expression levels found in microarray analyses, we performed qRT-PCR of APOD using 8 of the 16 mRNAs that had been used for the microarray hybridizations. APOD expression values as measured by microarrays were well correlated with expression values of qRT-PCR (Figure 14, R=0.93).

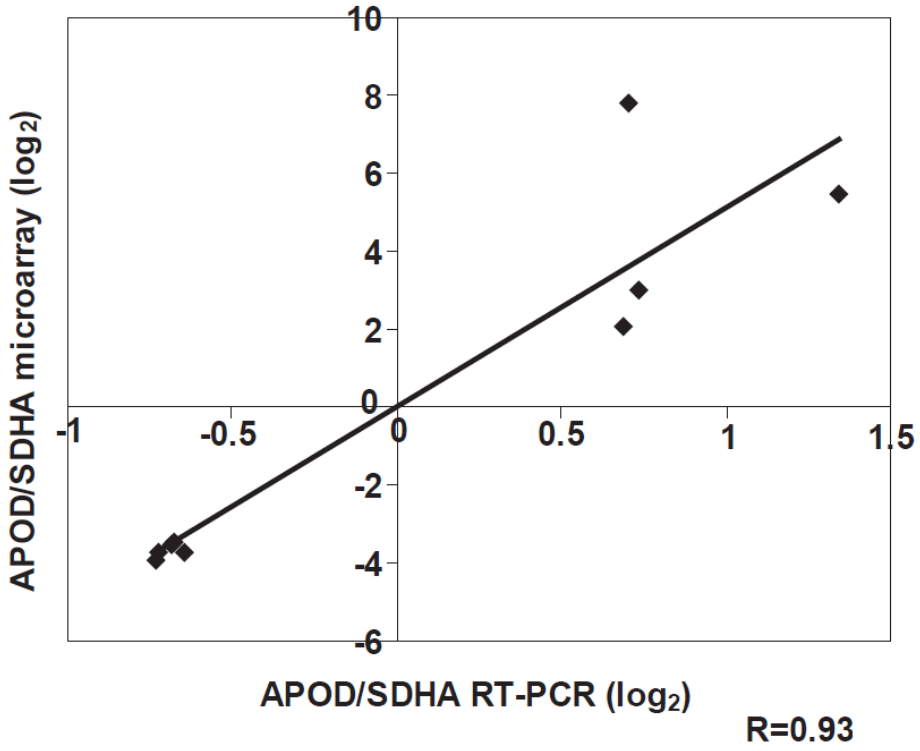

Figure 14. Correlation of APOD expression as measured by microarray and qRT-PCR (experimental set 1)

Expression values of microarray data (ratio of APOD divided through SDHA; Y-axis) versus quantitative real time PCR (qRT-PCR) (ratio of APOD divided through SDHA; X- axis). The correlation coefficient (R) is 0.93.

3.2.1 DHT mediated APOD expression in independent GSF strains

To further validate the inducibility of APOD, we carried out independent new cell culture experiments and DHT-treatments of 6 new scrotal fibroblast strains (S3, S5, S8, S9, S12, S13), 3 new CAIS strains (ARD682, ARD1144, ARD402), 6 PAIS strains (ARD446, ARD534, ARD001, ARD084, ARD377, ARD659) and 2 17ß-HSDIII deficiency strains (ARD111, ARD1373) (experimental set 2) (see also Table 2). QRT-PCR showed upregulation of APOD to 13.5±8.2 -fold (mean±SD) in 5 of the 6 control strains, while one of the control strains (S8) showed little or no relevant DHT-mediated APOD-upregulation (1.28 fold) (Figure 15). Intriguingly, when we sequenced the AR-gene of the S8-fibroblast strain (Table 2) without APOD response, we detected an inactivating I841S mutation, suggesting that this strain held a clinically occult mutation identical to a mutation our group had identified in a PAIS patient previously (Hiort O *et al.* 1996). We therefore had to exclude this sample from our analysis as part of the normal controls. The 3 new CAIS strains showed no obvious response of APOD to DHT treatment (1.2±0.7 fold) (Figure 15). In the 6 PAIS fibroblast samples, we observed a comparably slight upregulation of APOD (2±1.3 fold) (Figure 15). The differences between the normal scrotum and CAIS fibroblasts and between the normal scrotum and PAIS fibroblasts were statistically significant (p= 0.028 and 0.034, respectively; t-test). 17ß-HSDIII fibroblast strains showed APOD-upregulation to 9.96±1.4 fold in response to DHT and there was no significant difference compared to normal scrotal cells (p= 0.401; t-test) (Figure 15).

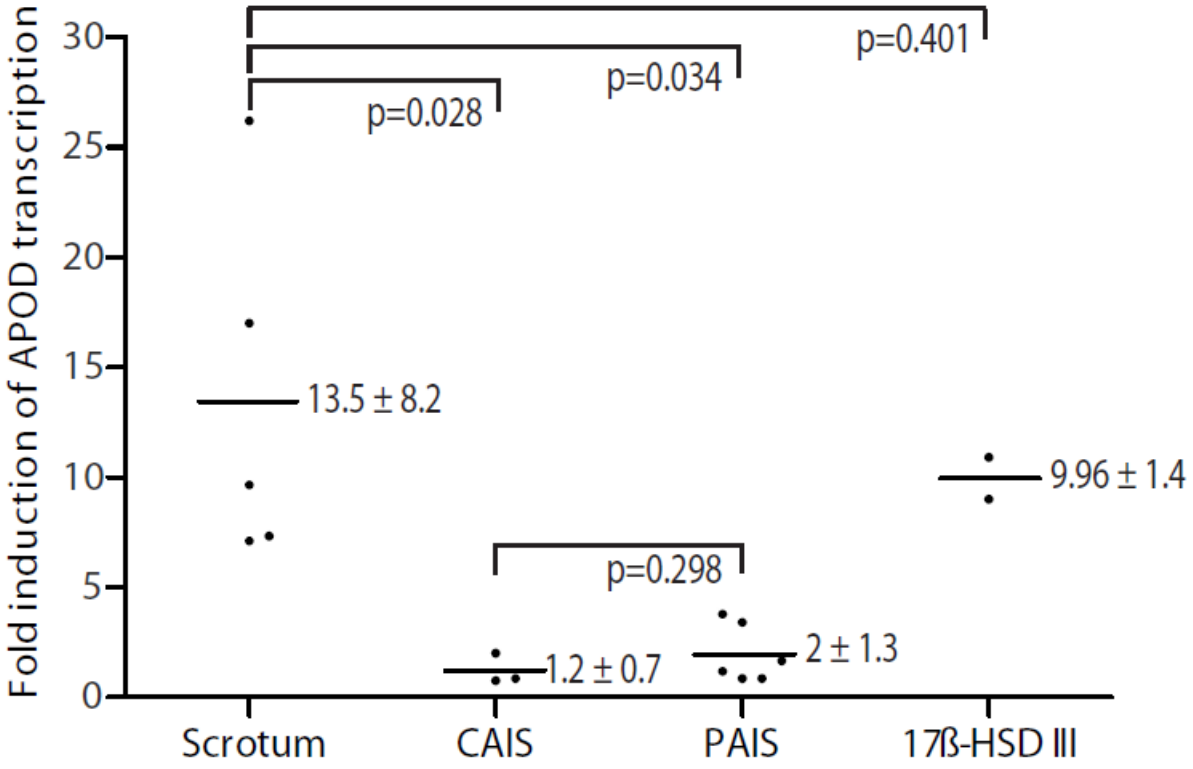

Figure 15. DHT-mediated induction of APOD transcription in normal male scrotum, PAIS, CAIS and individuals with 17ß-HSDIII defect (experimental set 2).

The Y-axis represents the fold induction of APOD transcription. The X-axis represents the different groups of investigated cell strains. Black dots represent the APOD induction in each of the normal scrotum (5 individuals), CAIS (3 individuals), PAIS (6 individuals) and 17ß-HSDIII deficiency (2 individuals) cell strains. The black lines represent the mean fold induction of APOD transcription per group based on these values.

3.2.2 Supplementary information

Microarray data can be accessed in the GEO data base (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lnctdkqeqgeouvc&acc=GSE11847).

3.3 DHT mediated gene expression patterns in human PBMCs

Unsupervised hierarchical clustering analysis of gene expression after 24 hrs of DHT stimulation on blood obtained at two different time points (10 A.M, 1 P.M) in 12 microarrays (experimental set 3) resulted in 651 transcripts. These transcripts demonstrated distinct, reproducible patterns of gene expression (Figure 16).

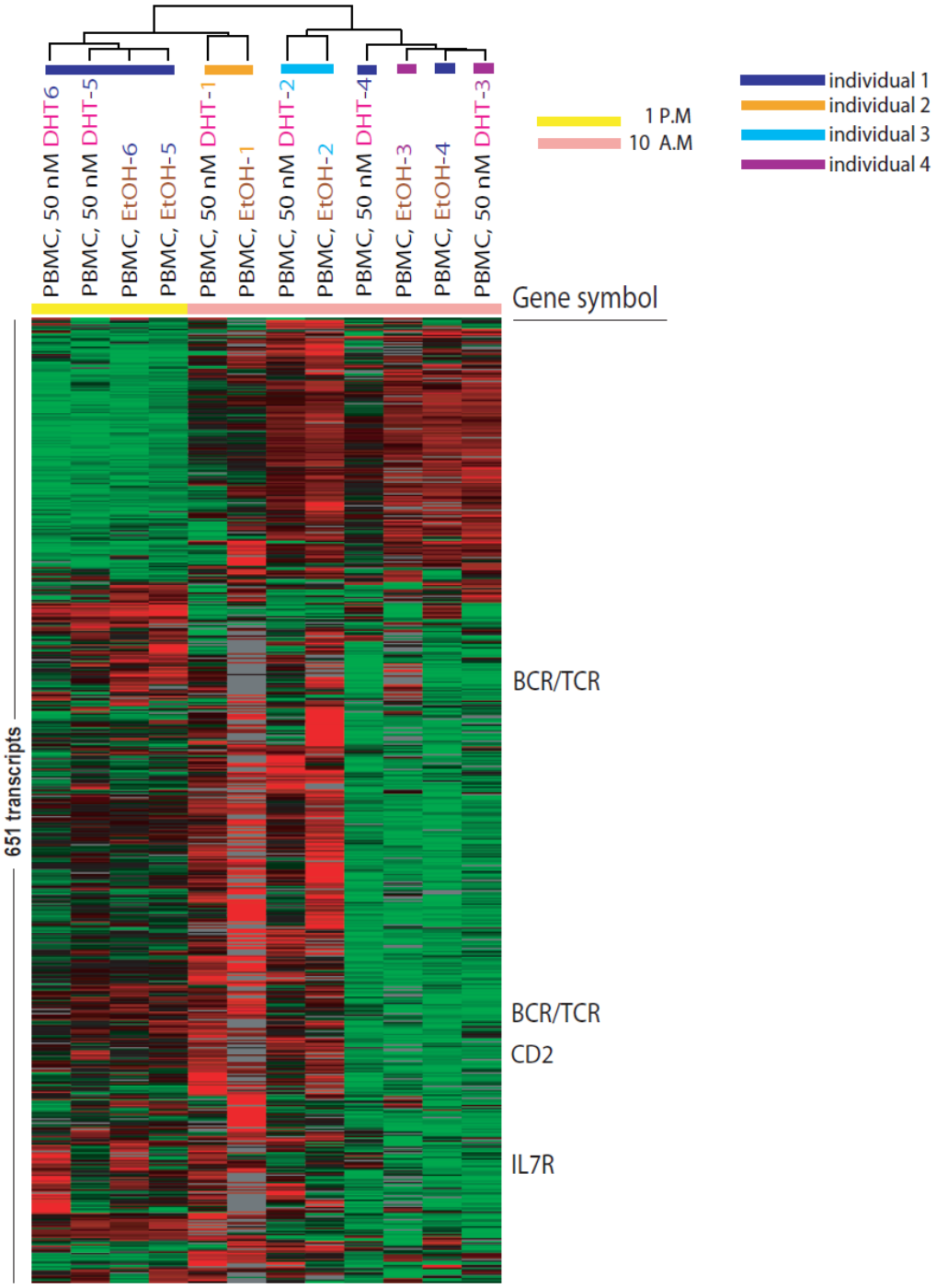

Figure 16. Unsupervised hierarchical cluster analysis of PBMCs treated with DHT, EtOH, obtained at 10 A.M and 1 P.M (experimental set 3). The heatmap represents

hierarchical clustering of 651 transcripts which had at least 80% interpretable data across the 12 microarrays and whose expression levels were at least 1-fold different from the mean expression level across all samples in at least 5 experiments. Transcripts are represented in rows and experiments in columns. Expression values per gene are centered by the mean $\log_2$ Cy5/Cy3 normalized ratio. Increasing red intensity corresponds to higher relative transcript levels compared to the mean expression level across all experiments. Increasing green intensity corresponds to relatively decreased transcript levels compared to the mean. Microarray experiments and genes were organized by hierarchical clustering using Pearson correlation metric and average linkage clustering (Eisen *et al.* 1998). TreeView software (Eisen *et al.* 1998) was applied to visualize the gene expression patterns of 651 genes in 12 PBMCs samples. The length of the arms of the dendogram reflects the degree of correlation between the samples.

Clustering sorted the microarray experiments according to the time of the blood obtained, but independent from the treatment of the cells. Mostly, individual specific PBMCs samples clustered together. Interestingly, the 10 A.M and 1 P.M PBMCs samples clustered together respectively, suggesting that the time point of blood samples influenced gene expression. Our unsupervised hierarchial clustering analysis revealed no significant changes of DHT treatment (Figure 16).

4. Discussion

The aim of this study was to identify androgen induced genes in primary genital skin fibroblasts of 46,XY individuals and PBMCs from healthy donors (46,XY) . Interestingly, for the first time I identified scrotum cells as a model cell strain in identifying androgen regulated gene expression in a primary human cell culture strain. Importantly, for the first time I identified APOD and two other transcripts as androgen induced in primary genital skin fibroblasts. Moreover, I analyzed APOD expression in GSFs of healthy males, different forms of AIS (CAIS, PAIS) and 17ß-HSDIII deficient patients.

4.1 The cell culture system

Foreskin fibroblast strains failed to qualify as a 'normal control' for androgen induced gene expression because they either did not show androgen induced response or showed inconsistent results (Berkovitz GD *et al.* 1996, Stillman SC *et al.* 1991, Nitsche EM *et al.* 1996, Holterhus PM *et al.* 2003, Bebermeier JH *et al.* 2006). Therefore, for the first time we tested scrotum cells as a 'normal control' in identifying androgen induced gene transcription. Scrotum cells have normal AR gene and express a normal AR, whereas 46,XY CAIS labia majora cells have inactivating mutations in the AR gene and 46,XY PAIS labia majora cells have partial inactivating mutations of the AR gene. The AR gene is normal in 17ß-HSDIII deficient patients. Scrotum, 46,XY labia majora and 17ß-HSDIII deficient fibroblasts were obtained from healthy and diseased patients, consequently.

4.2 Influence of RNA integrity and purity for microarray analysis

Reverse transcription of degraded RNA results in improper sequence of the cDNA transcripts, which may finally lead to unspecific hybridization of microarrays (D. Guo *et al*, 2004). The purity of RNA sample is based on the 28:18S ratio ($A_{260/280}$ 1.8 to 2.0) and gel electrophoresis is a standard procedure to determine the intact nature of RNA. Now the technology has advanced and more parameters are analyzed with the help of RNA nano chips (Agilent). These chips require 1 µl of RNA and provides the information of RNA ratio and RNA integrity number (RIN) ranging from 1 to 10 while 1 is defined as degraded RNA and 10 as the high intact RNA. RNA ranging from 7 to 10 is highly suitable for the microarray

experiments. Moreover, these chips also gives the information of the gel bands of 18S as smaller and 28S RNA as double in size like in gel electrophoresis. The ideal gel band of 28S RNA looks as double in size to 18S RNA. These chips provide two graphs for 18 and 28S RNA for the quality control purposes.

4.3 Microarray technology

Microarrays are highly demanding due to their potential in accurately measuring global gene expression changes. The use of different fluorescent dyes (such as Cy3 and Cy5) allow mRNAs from two different cell populations or tissues to be labelled with different colors, mixed and hybridized on the same array, which results in competitive binding of the target to the arrayed sequences (Duggan DJ *et al*. 1999). After hybridization and washing, the slide is scanned using two different wavelengths which is corresponding to the dyes used. Then the intensity of the same spot in both channels is compared. This results in a measurement of the ratio of transcript levels for each gene represented on the array.

4.3.1 Analysis of microarray gene expression data

The analysis of microarray gene expression requies careful image quantification, image corretion, normalization, data processing and visualizing tools. Statistical analysis of many microarrays requires multiple computers with high configuration. To solve this problem microarray data are generally uploaded to a server such as Stanford Microarray Database (SMD). Then the data can be filtered, and retrieved by applying different statistical strings. Some of the microarray analysis softwares used for finding the candidate gene analysis are Genepix, Significance of microanalysis (SAM), Cluster, Tree view, SMD and Scanalyze etc.

4.3.2 Microarray biases

The accuracy of microarray data depends upon the the precision, sensitivity and reproducibility of the measured values for each gene on the array.

Biological variation- It may be introduced by individual genetic and environmental factors as well as pooled samples (Churchill GA *et al.* 2002). *Experimental variation* depends upon the

following factors like RNA isolation procedure, RNA integrity, purity, batch of RNA amplification, dye incorporation and hybridization of samples (Franssen-van Hal NLW *et al.* 2002). The spotting, deposition of cDNA templates may cause biases (Diehl F *et al.* 2001). *Measurement variation* is associated with interference in measuring the laser signals due to high concentrations of Ozone present in the environment (may reduce the Cy5 signals), dust on the arrays, bubbles and high background.

4.3.3 Reproducibility of the microarrays

Microarrays are reproducible as the correlation of observed ratios between duplicate spots (clones) on a single microarray typically exceeds 95%. However, if the same sample is divided and hybridized to two different microarrays the correlation of the spot is approximately in the range of 60 to 80% and a little bit less in case of dye swapping (Churchill GA *et al.* 2002). Microarray replication may give illusory increase of statistical power. However, this may be due to the influence of particular experimental condition on a single individual. Therefore, it is ideal to analyze the experimental conditions in more individuals, which give the statistical significance of experimental conditions. Pooling the samples may decrease the biological sample variation, but it depends upon the sample handling like RNA isolation, RNA quality and florescence measurement error.

Amplification of universal common reference RNA in a single batch enough for a project, decreases the experimental biases. The replication of a single clone (spot) on the same microarray is highly advantageous as it gives increased precision and minimizes the artifacts like local high background, scratches and dust. Internal control spots (Stratagene Spiking control kit) can be advantageous for data analysis like calibration curve in determining the limits of detection, linear range, data saturation. However the control spots may vary from different microarrays and subjected to random noise. These control spots are either artificial genes or genetic material from unrelated organism.

Use of a single batch microarrays, which means microarrays printed in similar conditions may minimize the additional biases of microarrays, although there might be some unavoidable print tip effects.

4.3.4 Gene Expression Omnibus (GEO)

The National Center for Biotechnology Information (NCBI) has launched the GEO as a microarray data repository which is available to public and is used to analyze and retrieve the data. The data in GEO is generally derived from vast amount of multiple microarray experiments which are filtered and arranged by the biostatical softwares and can be used for functional genomics. GEO facilitates the option of comparing different microarray data obtained form different techniques and technologies. Creation of GEO or a website is necessary to publish a manuscript consisting of microarray experiments. Microarray Markup Language (MAML) group with NCBI group developed Microarray Gene Expression Database (MGED). The aim of this group was to establish a standard microarray data platform which is capable of data normalization, standard experimental controls, gene expression differences from different sources, and data analysis softwares. GEO is not yet available in MAML format.

4.3.5 Validation of microarray gene expression data

Independent confirmation of microarray data is achieved by using two different approaches: in silico analysis and the laboratory based analysis (Chuaqui RF *et al*. 2002). The in silico analysis method uses the available literature and databases. This approach is more useful with 'minimal information about a microarray experiment' (MIAME).

Laboratory based microarray data validation uses qRT-PCR, northern blot and immunohistochemistry (tissue microarrays). However, it is general practice to analyze the gene transcripts at protein level by using the Western blott analysis and Enzyme-Linked ImmunoSorbent Assay (ELISA) and protein microarray.

4.4 Fibroblast project: genome-wide microarray analysis

To date, no androgen induced genes have been identified in primary human genital skin fibroblasts. Using a genome-wide microarray strategy, we demonstrated for the first time that cultured normal male scrotum skin fibroblasts are transcriptionally responsive to DHT treatment *in vitro*. We found that APOD, a documented androgen regulated gene in the prostate cancer cell line LNCaP (Simard J *et al*. 1991), is significantly induced by DHT in

these cells. The presence of steroid hormone regulatory elements in the APOD gene promoter (Lambert J *et al*. 1993) corresponds with our findings.

4.4.1 APOD expression differences in scrotum, AIS and 17ß-HSDIII deficiency individuals

Severe reduction or complete absence of APOD induction in PAIS and CAIS samples, as assessed by qRT-PCR, support specificity of DHT-mediated APOD upregulation for the AR signaling pathway in the genital skin fibroblasts. High APOD response to DHT treatment in labia majora fibroblast strains derived from two different females with 17ß-HSDIII deficiency (Table 2) but intact AR confirmed that the AR-status but not labial versus scrotal anatomy per se is the determinant of APOD response to DHT in fibroblasts. Therefore, qRT PCR of DHT-induced APOD transcription in labiscrotal fibroblasts is a first molecular strategy for the functional identification of AIS assessing the degree of AR-mediated transcription activation in a primary human tissue culture model.

Our data on *scrotum* fibroblasts contrast with our previous observations in *foreskin* fibroblasts which failed to show a reproducible androgen response (Holterhus PM *et al*. 2003, Bebermeier JH *et al*. 2006). However, foreskin fibroblasts and scrotal skin fibroblasts derive from different embryonic origin within the bipotent external genitalia and differ significantly in their post natal baseline expression signatures (Holterhus PM *et al*. 2007). These differences in the baseline transcriptome might have contributed to the observed differences in androgen responsiveness.

4.4.2 Epigenetics of Sex differentiation

Epigenetic mechanisms such as gene methylation (Izbicka E *et al*. 1999) and chromatin structure modulation due to histone modification (Metzger E *et al*. 2005) play important roles during control of cell and tissue differentiation. As we initially suspected only marginal response of scrotum cells based on our previous experience with foreskin fibroblasts, we performed parallel experiments with the demethylating agent AZA. However,

AZA did neither affect DHT-induced APOD upregulation nor did it lead to the identification of additional androgen regulated genes silenced during development by methylation. We were surprised not to see significant changes in gene expression induced by AZA since we used doses that have been documented to change methylation in cell culture (Jüttermann R *et al*. 1994). A possible explanation of the lack of transcriptional changes due to AZA could be that we did not treat for sufficient time so that the number of cell divisions prior to confluency was inadequate to incur promoter demethylation.

4.4.3 APOD: A potential biomarker of AIS

Our data imply that APOD could be a potential 'biomarker' of androgen receptor function in genital tissues that could be used to establish the diagnosis of AIS. APOD response to DHT was extremely reduced or absent in all of our PAIS and CAIS samples, but was present in the 17ß-HSDIII deficiency fibroblasts. The utility of measuring the APOD response to DHT stimulation was suggested by the surprising finding of non-responsiveness in one control, S8. The S8 cell line had been considered to be derived from a normal male control because of phenotypic normal male external genitalia. This subject had initially come to medical attention because of infertility and scrotal fibroblasts had been harvested at the time of a TESE (testicular sperm extraction) surgical procedure. Interestingly, S8 fibroblasts showed severely reduced APOD responsiveness to DHT treatment and sequence analysis showed an inactivating I841S mutation within the ligand-binding domain of the AR-gene. We have previously described this mutation in a different patient having PAIS and ambiguous external genitalia (Hiort O *et al*. 1996). Therefore, reduced APOD transcription in S8 uncovered the diagnosis of AIS in this subject (MAIS = minimal androgen insensitivity syndrome) which was later proven by DNA sequence analysis. This surprising finding supports suitability of APOD as a potential molecular 'biomarker' for transcription regulatory function of the AR in the genital tissue.

APOD plays physiological roles in growth control and growth arrest (Simard J *et al*. 1991, Do Carmo S *et al*. 2002). High APOD expression in fibroblasts has been described in quiescent cells under conditions of confluency and serum-starvation (Provost PR *et al*. 1991). Interestingly, all four cultures of ARD411 showed higher baseline APOD expression on microarrays compared to the four ARD1097 experiments and to the untreated S4 and S15 experiments (Figure 12b). Since all these cells were cultured at comparable passage numbers

(Table 2), the baseline transcript level differences are not likely to reflect relevant differences in the state of senescence. Rather, we hypothesize that these differences reflect inter-individual differences in gene expression.

4.4.4 APOD: A pheromone transporter

APOD belongs to the alpha2mu-microglobulin superfamily, also called lipocalins, an evolutionary conserved superfamily of proteins that function in the binding and transport of many physiologically important ligands including pheromones (Flower DR *et al*. 1996). E-3-methyl-2-hexenoic acid (E-3M2H) is the most abundant axillary odor constituent in males and is carried to the skin surface by APOD before it is liberated from nonodorous apocrine secretions by axillary microorganisms (Zeng C *et al*. 1996). Pheromonal communication is an important pathway for the transmission of information on gender and social status in mammals (Bigiani A *et al*. 2005). There is evidence that male axillary pheromones can even mediate female menstrual synchrony by potential modulation of the hypothalamic pituitary-gonadal axis (Preti G *et al*. 1986). These considerations are intriguing in the context of androgen regulation of APOD-transcription in the human genital skin, AIS and disorders of sex development (DSD) but further studies are necessary to investigate the potential role of APOD therein.

4.5 Blood project

Peripheral blood may be the most feasible tissue source in clinical assessment of differences in gene expression patters due to the ease in accessibility. Blood is a complicated biological system consisting of different types of cells at different number and age. Blood gene expression profiling has been successfully employed in the diagnosis and classification of several diseases. Unfortunately, the blood transcriptome analysis is often challenging in several fields of biology and medicine. This is because of different parameters such as PBMCs isolation method, handling-washing, source and time.

4.5.1 Inter-individual and Intra-individual variation

The nature and extent of variation in inter-individual and intra-individual gene expression is an important issue in blood transcriptome. Blood consists of different types of cells and is the most variable tissue type in the body. The amount of blood cells often significantly varies from time to time and from subject to subject. For example, the normal range for monocytes in WBC is 1% to 5% and for lymphocytes is 20-50% (Harmening DM *et al.* 1997). This variation may influence the observed variation in genome-wide expression analysis (Fan H *et al.* 2005).

4.5.2 Genome-wide analysis of androgen induced genes in PBMCs

Using a genome-wide microarray strategy, we attempted in identifying the androgen induced genes in PBMCs obtained from healthy male donors at two different time intervals. Unsupervised hierarchical clustering analysis and SAM analysis could not provide DHT mediated gene expression patterns.

Hierarchical clustering analysis grouped the blood samples obtained at 10 A.M most closely as one group and 1 P.M as another group. This may be because gene expression differs at different time points. We tested two different time points because natural androgens and other hormone levels might influence the blood obtained at 10 A.M and the PBMCs may not respond to the *in vitro* DHT as they might have already reached the high threshold, which might be less at 1 P.M. We speculated that the individual specific and time dependent gene

expression patterns dominated disabling detection of observing the DHT mediated gene expression by the used microarrays. It was previously reported that medical history of the individuals, age, gender, Epigenetics, health status, metabolism, medications (if used any) may influence the transcriptome (Whitney AR *et al.* 2003, Harmening DM *et al.* 1997). The high polymorphic sequences of PBMCs might have high influence on individual variation in gene expression patterns and it may reflect underlying variation in their regulatory sequences, for example major histocompatibility complex (MHC) class II, human leukocyte antigen (HLA)-DQ and HLA-DR (Whitney AR *et al.* 2003). Fan H et al (2005) identified that several immunoglobin related transcripts are quite variable between different individuals.

The variability can be minimized by increasing the sample size, the time of the samples obtained and fasting before the blood withdrawl may minimize the noise between subjects (Fan H *et al.* 2005).

In conclusion, at this stage of our analysis PBMCs do not seem to be a good model in identifying androgen induced genes. Further investigation is required to understand the individual and inter-individual variation of gene expression of PBMCs in achieving the biomarkers of AIS.

5. Summary

Identifying androgen regulated genes by using genital skin fibroblasts

Mutations in the X-chromosomal AR-gene inhibit AR-function and in turn lead to the androgen insensitivity syndrome (AIS) in 46,XY individuals. The clinical spectrum of AIS ranges from complete AIS (CAIS) with normal female external genitalia due to complete inactivation of the AR to partial AIS (PAIS) with varying degrees of genital ambiguity including mild forms with only slight defects of virilization or isolated infertility caused by partial insufficiency of AR-function. Intriguingly, many patients with clinically evident AIS do not have documented mutations in the AR gene. Moreover, AIS patients with an established AR gene mutation, the correlation between genotype and phenotype is often poor, even within the same family. The aim of the present study was to identify androgen regulated genes that could provide opportunities for developing a bioassay for the diagnosis of AIS based on androgen receptor (AR) functional criteria.

In the first step, I used cDNA microarrays for a genome-wide screen for androgen-regulated genes in two normal male primary scrotal skin fibroblast strains compared to two labia majora fibroblast strains from 46,XY females with CAIS. Apolipoprotein D (APOD), Phosphoglucomutase 5 pseudogene 1 (PGM5P1) and Transcribed locus were significantly up-regulated by dihydrotestosterone (DHT) in scrotum fibroblasts while CAIS labia majora cells were unresponsive. Microarray data were well correlated with qRT-PCR (R=0.93).

Subsequently, I used qRT-PCR in independent new cell cultures and confirmed the significant DHT-dependent upregulation of APOD in five normal scrotum strains (13.5$\pm$8.2 (SD) fold) compared with three CAIS strains (1.2$\pm$0.7 fold, p=0.028; t-test) and six PAIS strains (2$\pm$1.3 fold, p=0.034; t-test). Moreover, two different 17ß-hydroxysteroid dehydrogenase III deficiency labia majora strains showed APOD induction in the range of normal scrotum (9.96$\pm$1.4 fold) supporting AR specificity.

Therefore, qRT-PCR of APOD mRNA transcription in primary cultures of labioscrotal skin fibroblasts is a promising tool for assessing AR function in AIS.

Identifying androgen regulated genes by using Peripheral blood mononuclear cells (PBMCs)

PBMCs have been an attractive tissue type for clinical research because it is easily obtained sample and can be successfully employed in disease prediction, classification, drug efficacy and disease prognosis. To date, no androgen induced genes were identified in AIS by using blood.

I used oligo microarrays for a genome-wide screening for androgen-regulated genes in 12 different samples of PBMCs. The blood was obtained from 4 different healthy males of different age and time points. Hierarchial clustering analysis and SAM did not provide androgen induction. In conclusion, at this stage of our analysis PBMCs do not seem to be a good model in identifying androgen induced genes.

5. Zusammenfassung

Identifikation androgenregulierter Gene am Beispiel kultivierter Genitalhautfibroblasten

Mutationen des X-chromosomalen Androgenrezeptorgens beeinträchtigen die Androgenrezeptorfunktion und führen deshalb zum Androgenresistenzsyndrom (androgen insensitivity syndrome, AIS) bei genetisch männlichen (46,XY) Individuen. Das klinische Spektrum der Androgenresistenz reicht von der kompletten Androgenresistenz mit normal weiblichem äußeren Genitale durch eine komplette Inaktivierung des Androgenrezeptors bis hin zur partiellen Androgenresistenz, bei der variable Ausprägungen der Virilisierung des äußeren Genitales beobachtet werden. Dies beinhaltet auch milde Formen mit einer nur leichten Untervirilisierung oder eine alleinige Infertilität. Bemerkenswert ist, dass viele Patienten mit klinisch eindeutigem Androgenresistenzsyndrom keine nachweisbaren Mutationen im Androgenrezpetorgen aufweisen. Darüber hinaus wird beobachtet, dass Patienten mit einer bekannten Androgenrezeptorgenmutation keine gute Genotyp-Phänotyp-Korrelation aufweisen, selbst bei Auftreten der gleichen Mutation in der gleichen Familie. Das Ziel der vorliegenden Studie war die Identifikation androgenregulierter Gene, die neue Möglichkeiten für die Entwicklung eines funktionellen Bioassays für die Diagnose Androgenresistenz eröffnen würden.

Im ersten Schritt habe ich cDNA Microarrays für ein genomweites Screening auf androgenregulierte Gene in zwei Zellkulturlinien normaler primärer Skrotalhautfibroblasten im Vergleich mit zwei Zellkulturlinien von Labia majora Fibroblasten von 46,XY-Frauen mit kompletter Androgenresistenz verwendet. Apolipoprotein D (APOD), Phosphoglucomutase 5 Pseudogene 1 (PGM5P1) und Transcribed locus waren in ihrer Transcriptionsrate signifikant durch Dihydrotestosteronbehandlung in skrotalen Fibroblasten heraufreguliert während Labia majora Fibroblasten von kompletter Androgenresistenz unresponsiv waren. Die Microarraydaten korrelierten sehr gut mit den qRT-PCR-Daten (R = 0,93).

Anschließend habe ich mit Hilfe der qRT-PCR die signifikante dihydrotestosteronabhängige Heraufregulation von Apolipoprotein D in 5 normalen Skrotalzelllinien ((13,5 ± 8,2 SD) fach) verglichen mit 3 Zelllinien von kompletter Androgenresistenz ((1,2 ± 0,7 SD) fach, p = 0,028; t-test) und 6 Zelllinien von partieller Androgenresistenz ((2 ± 1,3 SD) fach, p = 0,034; t-test) unabhängig überprüft. Darüber

hinaus zeigten zwei unterschiedliche Labia majora Zelllinien von Patienten mit 17ß-Hydroxysteroid-Dehydrogenase Typ III-Defizienz eine APOD-Induktion, die im Bereich derer von normalen Skrotalzelllinien lagen (9,96 ± 1,4-fach).

Deshalb stellt die qRT-PCR der APOD mRNA-Transcription in Primärkulturen labioskrotaler Hautfibroblasten ein vielversprechendes Werkzeug für die zukünftige Untersuchung der Androgenrezeptorfunktion bei Androgenresistenz dar.

Identifizierung von Androgen-regulierten Genen in mononukleären Zellen des peripheren Blutes (PBMC)

PBMC stellen eine attraktive Gewebeart für die klinische Forschung dar, aufgrund der relativ einfachen Probengewinnung und der Anwendung in der Krankheitvorhersage, Klassifikation, Drogenwirksamkeit und Krankheitsprognose. Bislang wurden im Blut keine Androgen-induzierten Gene bei AIS identifiziert.

Daher wurden Oligo-Microarrays zur Untersuchung des Genoms bezüglich Androgen-regulierter Gene in 12 verschiedenen PBMC Proben verwendet. Das Blut stammte von 4 männlichen, gesunden Probanden unterschiedlichen Alters, wobei die Proben zu verschiedenen Zeitpunkten entnommen wurden. Hierarchische Clusteranalyse und SAM ergaben keine Induktion von Androgen. Zusammenfassend stellt die Analyse durch PBMC kein gutes Modell zur Identifizierung von Androgen-induzierten Genen dar.

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7. Appendix

7.1 Abbreviations

APOD	Apolipoprotein D
AZA	5'-aza-deoxy-cytidine
AIS	Androgen insensitivity syndrome
AR	Androgen receptor
ARE	Androgen response elements
aRNA	antisense RNA
aa	amino acids
BSA	bovine serum albumin
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CAIS	Complete Androgen insensitivity syndrome
Cy5/Cy3	Cyanine dye5, Cyanine dye3
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSD	Disorders of sex development
ddNTP	Dideoxyribonucleotide triphosphate
E-3M2H	E-3-methyl-2-hexenoic acid
EtOH	Ethanol
EDTA	Ethylene diamine tetra acetic acid
FBS	Fetal bovine serum
GSF	Genital skin fibroblasts
gm	grams
hr(s)	hour(s)
HRE	Hormone response elements
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IL	Interleukin
kb	kilobases

kDa	kiloDalton
LNCaP	human prostate carcinoma
M	molar
mg	milli grams
min	minute(s)
ml	milliliter
mM	millimolar
mRNA	messenger RNA
MAIS	minimal androgen insensitivity syndrome
ng	Nanograms
PBS	Phosphate buffered saline
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase chain reaction
PGM5P1	Phosphoglucomutase 5 pseudogene 1
PAIS	Partial androgen insensitivity syndrome
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SHBG	Sex hormone-binding globulin
SAM	Significance Analysis of Microarrays
SDHA	Succinate Dehydrogenase Subunit A
SDS	Sodium dodecyl sulfate
SFGF	Stanford Functional Genomics Facility
T	Testosterone
TIF2	Transcriptional intermediary factor 2
TESE	Testicular sperm extraction
UV	Ultra violet
°C	degrees centigrade
µg	micrograms
µl	micro litre
µM	micro molar
17ß-HSDIII	17ß-hydroxysteroid dehydrogenase III

7.2 Index of Figures

7.3 Index of Tables

8. Curriculum vitae

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Holterhus PM, Bebermeier JH, Werner R, Demeter J, Richter-Unruh A, Appari M, Riepe F, Brooks JD, Hiort O. Dissection of the genetic and hormonal sex of blood in disorders of sex development (DSD). BMC Genomics. 2009 Jul 1;10(1):292. [Epub ahead of print]

Abstracts and Posters:

- Apolipoprotein D (APOD) is a potential biomarker of androgen insensitivity syndrome (AIS), 52nd Symposium of the German Society of Endocrinology (DGE), 2009, Giessen, Germany.

- Appari M, Werner R, Hiort O, Cario G, Hiort O, Riepe F, Holterhus PM. 2008 Variability and specificity of dihydrotestosterone-induced apolipoprotein D transcription in labioscrotal derived human skin fibroblasts. Horm Res 70 (Suppl 1), Oral presentation at European Society for Paediatric Endocrinology (ESPE), Istanbul, Turkey.

- Apolipoprotein D (APOD) is an androgen-regulated gene in normal male scrotal fibroblasts in the androgen insensitivity syndrome (AIS). Oral presentation at 24th German Pediatric Endocrinology (APE), 2007, Mainz, Germany.

- Appari M, Karvelis B, Hohmann G, Cario G, Werner R, Hiort O, Riepe F, Holterhus PM (2007). Apolipoprotein D (APOD) is an androgen-regulated gene in normal male scrotal fibroblasts. Horm Res 68 (Suppl 1): 1-282 (Abstract and poster, ESPE 2007, Helsinki)

- Brämswig N, Appari M, Baus I, Welzel M, Sippell WG, Riepe FG, Holterhus PM (2007). Genome-wide analysis of GH responsive genes in PBMC is a potential technique to characterize future biomarkers for growth prediction. Horm Res 68 (S1) 1-282 (Abstract and poster, ESPE 2007, Helsinki)

- Holterhus PM, Bebermeier JH, Appari M, Werner R, Richter-Unruh A, Riepe F, Hiort O. Genome-wide transcriptome analysis of peripheral blood mononuclear cells (PBMC) reveals male, female and ambiguous gene transcription signatures. Horm Res 2007;68 (S1): 1-282 (Abstract, ESPE 2007, Helsinki)

9. Statement of Originality

I declare that this thesis has not been submitted in any form for another degree or diploma at any university. Results discussed in this thesis are my own work, and information derived from the literature or unpublished work of others has been acknowledged in the text and a list of references provided.

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel von mir eingesetzt worden sind. Des weiteren versichere ich, dass die vorliegende Dissertation weder ganz noch zum Teil bei einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegen hat.

Kiel, 18.5.2009

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Mahesh Appari