AN INVESTIGATION INTO THE METABOLISM OF CLASSIC AND 11-OXYGENATED ANDROGENS IN PERIPHERAL BLOOD MONONUCLEAR CELLS

by

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Abstract

In both sexes, androgens are responsible for the initiation and maintenance of sexual differentiation and reproduction, as well as the development of secondary male characteristics including male pattern body hair, muscle bulk, sexual function and reproductive capacity. Androgens are also key regulators of immune cell function and have a suppressive influence on differentiation, proliferation, and cytokine production. Local generation of active androgens from circulating androgen precursors is an essential mediator of androgen action in peripheral androgen target cells and tissues. To examine the activation of classic and 11-oxygenated androgens within human peripheral blood mononuclear cells (PBMCs), PBMCs were isolated from healthy donors and subsequent activity analysis assays were carried out. PBMCs were incubated *ex vivo* with androgen precursors and active androgens from the classic pathway (DHEA, A4 and T) and the 11-oxygenated androgen pathway (11KA4, 11KT, 11OHA4 and 11OHT). In total, 12 steroids were quantified in 50% (v/v) methanol in water by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The expression of genes encoding steroidogenic enzymes was assessed by quantitative PCR (qPCR). qPCR showed that the enzyme AKR1C3, rather than HSD17B3, was the major reductive 17β-hydroxysteroid dehydrogenase in PBMCs, responsible for the activation of the 11-oxygenated androgen 11-ketotestosterone and the classic androgen testosterone. Approximately 8-fold more 11-ketotestosterone than testosterone was produced from their respective precursors, indicating preferential activation of 11-oxygenated androgens by AKR1C3. These results identified 11-ketotestosterone as the major active androgen in PBMCs. RNAseq analysis of FACS-sorted PBMC subpopulations (dice-database.org) revealed natural killer cells to be the major location of AKR1C3 activity and expression. The enzyme SRD5A1 catalysed the 5α-reduction of classic, but not 11-oxygenated androgens in PBMCs. Overall, this project has revealed that 11-oxygenated androgens are the favoured substrate for AKR1C3 in PBMCs, predominantly due to natural killer cell AKR1C3 activity, producing the major active androgen 11-ketotestosterone.

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Declarations

I declare this thesis is my own work. The examination copy is an exact copy of the copy submitted to Turnitin.

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11KA4	11-ketoandrostenedione
11KT	11-ketotestosterone
11KDHT	11-keto-5α-dihydrotestosterone
11OHA4	11β-hydroxyandrostenedione
11OHT	11β-hydroxytestosterone
17OHP	17α-hydroxyprogesterone
17OHPreg	17α-hydroxypregnenolone
A4	Androstenedione
ACTH	Adrenocorticotropic hormone
AKR1C3	Aldo-keto reductase family 1 member C3

AR	Androgen receptor
b_5	Cytochrome *b*5
BMI	Body mass index
CAH	Congenital adrenal hyperplasia
CRPC	Castration resistant prostate cancer
CYP	Cytochrome P450
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	5α-dihydrotestosterone
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
HPA	Hypothalamic-pituitary-adrenal
HSD11B1	11β-hydroxysteroid dehydrogenase type 1
HSD11B2	11β-hydroxysteroid dehydrogenase type 2
HSD17B	17β-hydroxysteroid dehydrogenase
HSD17B3	17β-hydroxysteroid dehydrogenase type 2
HSD17B3	17β-hydroxysteroid dehydrogenase type 3
HSD17B4	17β-hydroxysteroid dehydrogenase type 4
HSD3B1	3β-hydroxysteroid dehydrogenase type 1
HSDs	Hydroxysteroid dehydrogenases
IL12A	Interleukin 12A
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LH	Luteinizing hormone
NADPH	Nicotinamide adenine dinucleotide phosphate
NIH	National Institute of Health

PAPSS2	PAPS synthase 2
PCOS	Polycystic ovary syndrome
POR	Cytochrome P450 oxidoreductase
SDRs	Short chain dehydrogenases
SHBG	Sex hormone binding globulin
SRD5A1	5α-reductase type 1
SRD5A2	5α-reductase type 2
StAR	Steroidogenic acute regulatory protein
STS	Steroid sulphatase
SULT2A1	Sulphotransferase 2A1 enzyme (= DHEA Sulphotransferase)
T	Testosterone

1. Introduction

1.1 Steroid biosynthesis

1.1.1 Introduction to steroidogenesis

Steroids are lipophilic hormones which are vital for the regulation of the stress response, water and salt levels and the commencement and maintenance of sexual differentiation and reproduction (Hiller-Sturmhofel et al., 1998). In the adrenal cortex steroid hormones are produced by *de novo* steroidogenesis, a process tightly regulated by a combination of biochemical, cellular and hormonal mechanisms. It is of utmost importance to delineate the pathways of steroidogenesis in order to understand its associated diseases, such as disorders of sexual differentiation and reproduction.

Cholesterol is the precursor for the *de novo* biosynthesis of steroids and is derived from a variety of sources, with low-density lipoproteins from the diet being the main source for steroidogenesis (Bremer et al., 2014). Tropic stimulating hormones act via second messengers to induce the activation of steroidogenic acute regulatory protein (StAR) (Manna et al., 2016). StAR is an accessory protein which transfers cholesterol to the inner mitochondrial membrane. This process is the rate limiting step of *de novo* steroidogenesis (Manna et al., 2016). Following this, the sidechain of cholesterol is cleaved to produce pregnenolone, which is the universal precursor for the biosynthesis of all steroid hormones (Miller et al., 2011). The ability to convert significant amounts of cholesterol to pregnenolone is limited to cells within three primary steroidogenic organs: cells within the adrenal cortex, the Leydig cells of the testes and theca cells of the ovaries. Additionally, trophoblasts cells of the placenta can synthesise steroids *de novo* (Bremer et al., 2014; Miller et al., 2011).

1.1.2 Steroidogenic enzymes

Cytochromes P450 (CYP) enzymes are vital for steroidogenesis. The haeme-containing CYPs function as monooxygenases and utilise electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH), which are transferred to the CYP via specific electron transfer partners, to activate molecular oxygen (Miller et al., 2011). The enzymes then introduce one oxygen atom into the substrate, while the other oxygen atom is reduced to H_2O. Among other reactions, CYPs can catalyse hydroxylations and C-C bond cleavages of substrates, both of which are essential reactions for steroidogenesis (Payne et al., 2004).

CYPs can be divided into two groups based on their intracellular location and their distinct electron transfer systems required to transfer electrons from NADPH to the haeme (Hannenmann et al., 2016). Mitochondrial CYPs (CYP11A1, CYP11B1 and CYP11B2) are located within the inner mitochondrial membrane and depend on a class I redox system, while CYPs located in the membrane of the endoplasmic reticulum (CYP17A1, CYP19A1 and CYP21A2) depend on a class II redox system (Payne et al., 2004). In the class I redox system, the flavin adenine dinucleotide group of the ferredoxin reductase oxidises NADPH and transfers the electrons step-wise to the ferredoxin. The electrons are then delivered to the CYP which activates the molecular oxygen at the haeme iron (Bremer et al., 2014). The class II redox system of microsomal CYPs involves the electron donor enzyme cytochrome P450 oxidoreductase (POR), which contains two flavins. The electrons are transferred from NADPH via the flavin adenine dinucleotide (FAD) and the flavin mononucleotide (FMN) domains of POR to the CYP (Lin et al., 1993; Miller et al., 2011).

Of equal importance in steroidogenesis are the hydroxysteroid dehydrogenases (HSDs). These enzymes can reduce or oxidise steroid hormones via a hydride transfer mechanism which requires NAD(P)H and $NAD(P)^+$ cofactors (Bremer et al., 2014). HSDs can be subdivided into short chain dehydrogenases (SDR) and aldo-keto reductases (AKR) (Bremer et al.,2014). Although most HSD-catalysed reactions are bi-directional, there is a prominent directionality *in vivo*, which is determined

by factors including the availability of intracellular cofactors, the affinity for substrates and co-factors and the pH (Luu-The et al., 1991).

1.1.3 Adrenal steroid biosynthesis

The human adrenal cortex is responsible for the *de novo* biosynthesis of glucocorticoids, mineralocorticoids and androgen precursors, illustrated in **Figure 1.1**. The adrenal cortex can be subdivided into three zones which have differential expression of steroidogenic enzymes. This allows each zone to produce a distinct class of steroid hormones (Payne et al., 2004). The outer zone of the adrenal cortex, named zona glomerulosa, is regulated by the renin-angiotensin system and expresses enzymes responsible for the production of the mineralocorticoid aldosterone (Bremer et al., 2014;). The middle zone, named zona fasciculata, is responsible for the production of the glucocorticoid cortisol. The innermost zone, named zona reticularis, is responsible for the production of 19-carbon androgen precursors including dehydroepiandrosterone (DHEA), androstenedione (A4) and 11β-hydroxyandrostenedione (11OHA4) (Turcu et al., 2015).

The adrenal cortex can synthesise small amounts of active androgens, however the majority of adrenal androgen precursors are activated in peripheral tissue and gonads (Luu-The et al., 2013; Turcu et al., 2014). The biosynthesis of both adrenal androgen precursors and glucocorticoids within the human adrenal cortex is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Handa et al., 2014). In essence, corticotropin-releasing hormone is released by the hypothalamus, which causes corticotrope cells within the anterior pituitary to release adrenocorticotropic hormone (ACTH), which is the primary stimulus for the adrenal gland to produce DHEA and cortisol (Simpson et al., 1988; Xing et al., 2011). The release of cortisol results in negative feedback on the pituitary and hypothalamus, which inhibits any additional stimulation of the adrenal gland (Gjerstad et al., 2018). Contrastingly, there is no negative feedback on the HPA axis by the androgen precursors produced in the adrenal gland (Schiffer et al., 2019).

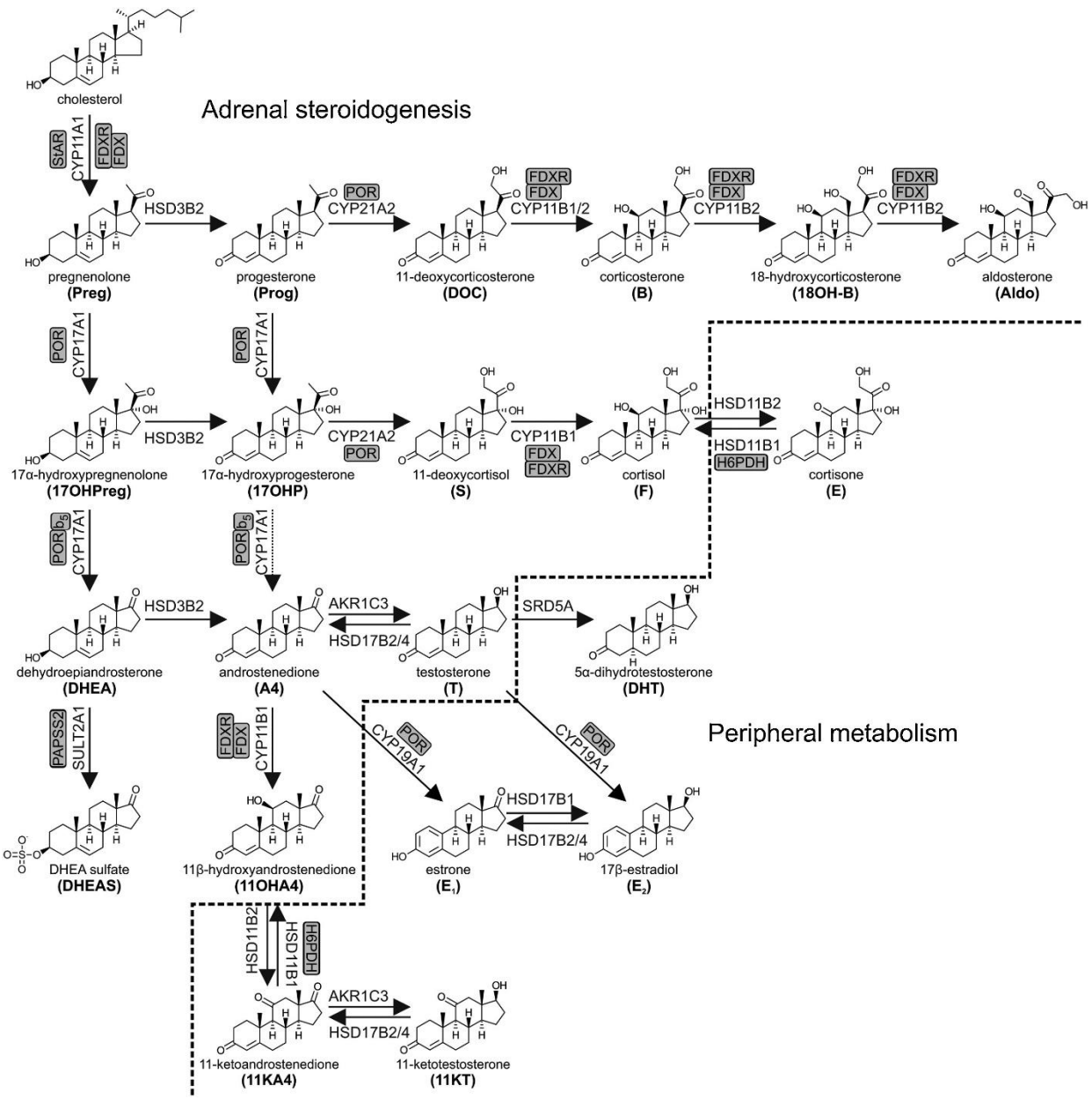

Figure 1.1 Schematic summary of steroidogenesis occurring in the adrenal cortex, along with modulation of steroid hormone activity by peripheral tissue. Via a series of biosynthetic steps, the steroid precursor pregnenolone can be converted to mineralocorticoids, glucocorticoids and androgens. Catalysing enzymes are labelled with arrows. All accessory proteins are labelled in boxes: steroidogenic acute regulatory protein (StAR), ferredoxin (FDX), ferredoxin reductase (FDXR), cytochrome b_5 (b_5), cytochrome P450 oxidoreductase (POR), hexose-6-phosphate dehydrogenase (H6PDH); PAPS synthase 2 (PAPSS2). Figure obtained from Schiffer et al., 2019.

1.1.4 Gonadal steroid biosynthesis

The increase in hypothalamic-pituitary-gonadal axis activity during puberty initiates gonadal steroidogenesis (Oyola et al., 2017). Within the testes and ovaries, steroidogenesis is mostly limited to the biosynthesis of 19-carbon androgens as well as 18-carbon oestrogens in females. Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus in a pulsatile rhythm, which stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary (Schiffer et al., 2019). LH and FSH then regulate gonadal steroidogenic activity. LH acts on ovarian theca cells and testicular Leydig cells and promotes the conversion of A4 to T, which is catalysed by 17β-hydroxysteroid dehydrogenase type 3 (HSD17B3) (Schiffer et al., 2019). In men, the majority of active testosterone (T) is produced and released from the Leydig cells, with lower levels of A4 and DHEA also released into circulation (Hammond et al., 1977; Ishida et al., 1990). In females, the majority of A4 diffuses into granulosa cells where it is converted to oestrone, oestradiol and oestrone sulphate, which is regulated by FSH (Skinner et al., 2018). The release of androgens and oestrogens causes negative feedback at the hypothalamus and the pituitary, respectively, which suppresses LH (Handa et al., 2014).

1.2 Androgen biosynthesis and metabolism

1.2.1 The classic androgen pathway

Most steroids produced by the adrenal glands are inactive androgen precursors, which become activated in peripheral target tissues (Turcu et al., 2015). Androgens can be produced via three interconnecting pathways: the classic androgen biosynthesis pathway, the 11-oxygenated androgen pathway and the alternative DHT biosynthesis pathway, which operate across multiple tissues and are illustrated in **Figure 1.2** (Schiffer et al., 2019). In the classic pathway, CYP17A1 is responsible for the conversion of pregnenolone to 17α-hydroxypregnenolone (17OHPreg), and the subsequent conversion of 17OHPreg to androgen precursor DHEA (Petrunak et al., 2014). The 17,20-lyase activity of CYP17A1 requires the presence of accessory protein cytochrome b_5 (*b_5)* in addition to electron transfer from POR (Auchus et al., 1998; Turcu et al., 2015). Following this, DHEA gets converted to

androgen precursor A4 by 3β-hydroxysteroid dehydrogenase activity (HSD3B). Activating, reductive 17β-hydroxysteroid dehydrogenase (HSD17B) activity is responsible for the conversion of inactive androgen A4 to active androgen T. The HSD17B isoform responsible for this activation in the adrenals and peripheral tissue is aldo-keto reductase family 1, member C3 (AKR1C3), also named 17β-hydroxysteroid dehydrogenase type 5 (Penning et al., 2000; Nakamura et al., 2009). This reaction is bi-directional, and T can become inactivated to A4 by oxidative 17β-hydroxysteroid dehydrogenase activity (Labrie et al., 1997). The HSD17B isoforms responsible for the inactivation of T are 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2) and 17β-hydroxysteroid dehydrogenase type 4 (HSD17B4) (Labrie et al., 1997). Active T can get converted to the most potent human androgen 5α-dihydrotestosterone (DHT) by steroid 5α-reductase (SRD5A) activity, which mostly takes place within peripheral target tissue (Thigpen et al., 1993;). In addition to the adrenal glands and peripheral tissue, both the ovarian theca cells and Leydig cells of the testes also follow the classic pathway where biosynthesis is focussed on producing active androgens T and DHT (Schiffer et al., 2019).

1.2.2 The alternative DHT biosynthesis pathway

In some circumstances, including during foetal development (Reisch et al., 2019) and certain pathological conditions such as CYP21A2 deficiency in congenital adrenal hyperplasia (CAH), an accumulation of progesterone and 17α-hydroxyprogesterone (17OHP) in circulation can result in the activation of an alternative DHT biosynthesis pathway (Arlt et al., 2004; Baranowski et al., 2018; Jones et al., 2017; Kamrath et al., 2011). Via a series of conversions by 3α-reductase activity of AKR1C enzymes and 17,20-lyase activity of CYP17A1, androsterone is synthesised from progesterone and 17OHP **(Figure 1.3).** Androsterone, which is usually considered an inactive metabolite, can then become activated to DHT by 17β-reduction and 3α-oxidase reactions (Bauman et al., 2006). It has been shown that androsterone is the main circulating androgen precursor for the alternative DHT biosynthesis pathway in the male foetus during the second trimester (O'Shaughnessy et al., 2019).

1.2.3 The 11-oxygenated androgen pathway

Following DHEA and DHEAS, the second most abundant androgen precursor produced in the human adrenal gland is 11OHA4 (Turcu et al., 2020). Within the zona reticularis, CYP11B1 catalyses the conversion of classic androgens to their 11β-hydroxyl derivatives, shown in **Figure 1.3.** Here, A4 is converted to 11OHA4, and to a more minor extent T to 11β-hydroxytestosterone (11OHT) (Swart et al., 2013). Due to the higher concentration of A4 compared to T in the zona reticularis, 11OHA4 is the major 11-oxygenated androgen precursor generated by CYP11B1 (Rege et al., 2013). In the adrenal glands, both 11OHA4 and 11OHT can then be converted to their 11-keto derivatives 11-ketoandrostendione (11KA4) and 11-ketoteststerone (11KT) by low levels of 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2). However, HSD11B2 is predominantly expressed in peripheral tissues, such as the kidneys, which is responsible for most of the conversion of adrenal 11OHA4 to 11KA4 (Turcu et al., 2020). The inactive precursor 11KA4 gets converted to the active androgen 11KT by AKR1C3, which mostly takes place in peripheral tissues. Like classic T, this reaction is bi-directional, and 11KT can become inactivated to 11KA4 by oxidative 17β-hydroxysteroid dehydrogenases activity by the isoforms HSD17B2 and HSD17B4 (Koh et al., 2002). 11KT can become further converted to active androgen 11-keto-5α-dihydrotestosterone (11KDHT) by SRD5A activity (Storbeck et al., 2013).

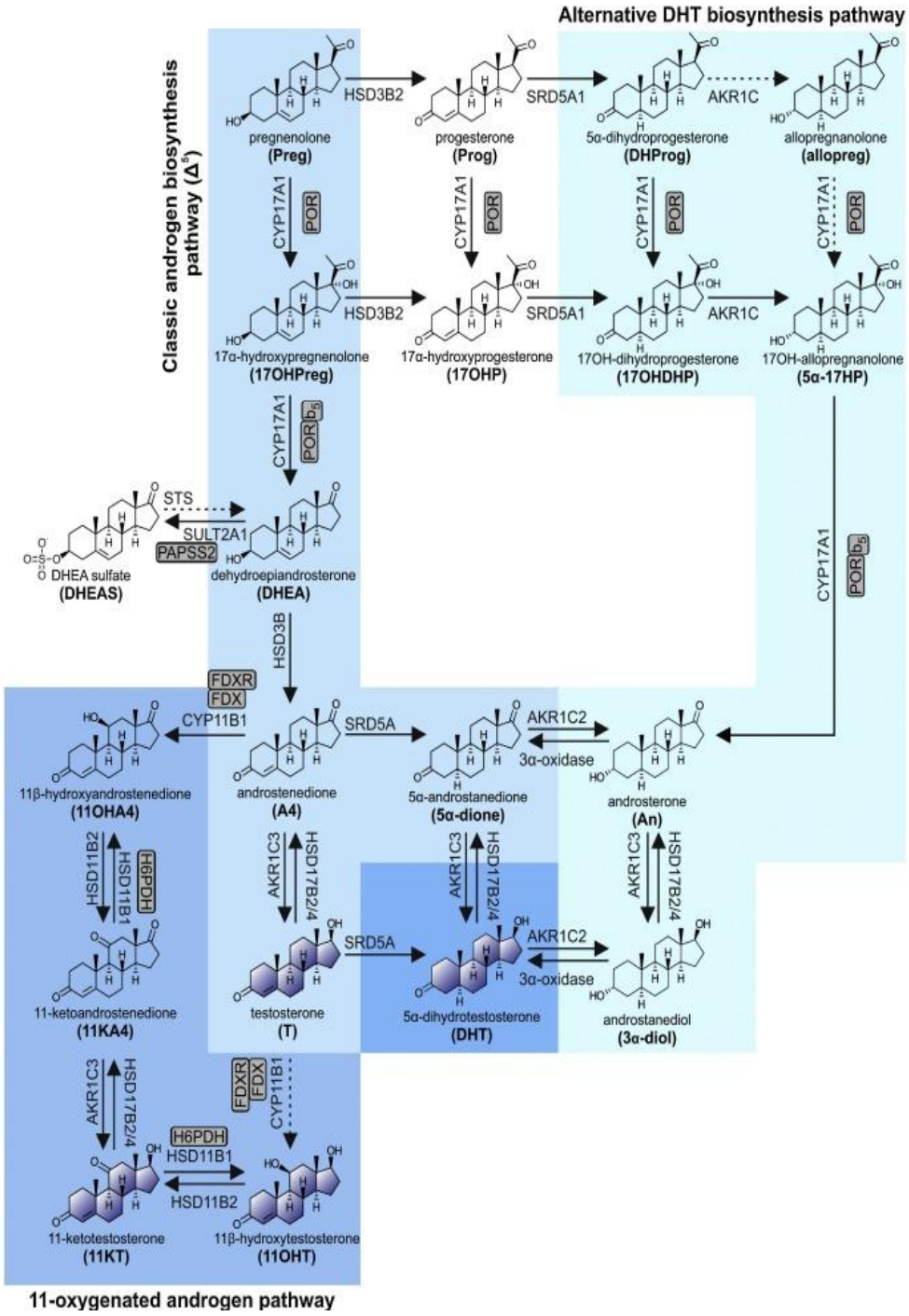

Figure 1.2 Schematic summary of androgen biosynthesis via three interconnected pathways operating across multiple tissues. Bioactive androgens can be generated by the classic (T and DHT), the alternative DHT biosynthesis pathway and the 11-oxygenated androgen pathway (11KT). The alternative DHT pathway bypasses to production of T to produce the more potent androgen DHT. Figure obtained from Schiffer et al., 2019.

1.2.4 The androgen receptor mediates the biological effects of androgens

The action of active androgens T, DHT, 11KT and 11KDHT is mediated via the androgen receptor (AR) (Davey et al, 2016; Pretorius et al., 2016). The AR is a ligand-dependent nuclear transcription factor and is part of the steroid hormone nuclear receptor family, which includes the oestrogen receptor, glucocorticoid receptor, mineralocorticoid receptor and progesterone receptor (Ben-Batalla et al., 2020). There are two distinct mechanisms of ligand-dependent AR action: DNA binding-dependent (genomic) and non-DNA binding-dependent (non-genomic) (Davey et al., 2016). In the DNA dependent pathway, the absence of an androgen ligand means the AR is situated in the cytoplasm and associated with heat-shock proteins (HSPs) and chaperone proteins (Tan et al., 2015). Androgen binding to the AR in the cytosol induces the dissociation of the AR and HSPs and leads to the subsequent translocation of the androgen/AR complex to the nucleus (Tan et al., 2015). Once the AR complex is transferred to the nucleus, ligand-activated AR binds to specific DNA regulatory sequences named androgen response elements (Davey et al., 2016). This ligand-dependent transcription factor modulates gene expression through direct DNA binding and the recruitment of several co-regulators to induce epigenetic histone modifications and chromatin remodelling at target genetic loci (Ben-Batalla et al., 2020; Davey et al., 2016; Miller et al., 2013). Using a promoter reported assay in transiently transfected COS-1 cells, it has been shown that 11KT binds and activates the androgen receptor (AR) with affinity, potency and efficacy comparable to T (Pretorius et al., 2016). Additionally, it has been shown 11KT activates the expression of AR-regulated genes and promotes cell growth using two androgen-dependent CRPC cell lines (Pretorius et al., 2016).

In both sexes, the main role of androgen action mediated via the AR is the initiation and maintenance of sexual differentiation and reproduction (Ben-Batalla et al., 2020). Androgens are also responsible for the development of secondary male characteristics such as male pattern body hair, muscle bulk, sexual function and reproductive capacity (Kempegowga et al., 2020). Androgens influence libido, general wellbeing and energy levels in both sexes, as well as muscle mass and bone mass in females (Tan et al., 2015; Kempegowda et al., 2020). Besides the reproductive organs, the AR has been shown

to be expressed in a diverse range of tissues and systems including bone, muscle and adipose tissue, as well as in the immune, cardiovascular and neural systems in which androgens have been documented to exert biological actions (Tan et al., 2015; Ben-Batalla et al., 2020).

1.2.5 Activation of circulating androgens in peripheral tissue

Androgens have crucial effects on peripheral target tissue, such as the regulation of lipid homeostasis and development of adipose tissue (Trigunaite et al., 2015; O'Reilly et al., 2017). For local androgen activation to take place, the respective peripheral target tissues must express the relevant steroidogenic enzymes in order to convert inactive androgen precursors to their active androgens **(Figure 1.3).** Circulating DHEA can be converted to A4 by peripheral 3β-hydroxysteroid dehydrogenase type 1 (HSD3B1). 11OHA4 produced by the human adrenal glands is converted to 11KA4 in the periphery, mostly by HSD11B2 in the kidneys (Turcu et al.,2020). Both A4 and 11KA4 are subsequently converted to T and 11KT, respectively, by peripheral AKR1C3 activity, this conversion has been shown to taken place in adipose tissue (Quinkler et al., 2004). In addition, AKR1C3 expression has been observed in a wide range of peripheral tissues including the prostate, mammary glands and uterus (Penning et al., 2000).

Of note, a recent study found that AKR1C3 transiently overexpressed in HEK293 cells has an eightfold higher catalytic efficiency when utilising 11KA4 as its substrate as opposed to A4 and confirmed that 11-oxygenated androgen precursors are the preferred substrate for AKR1C3 using different prostate cancer cell lines (Barnard et al., 2018). Within peripheral target tissue, SRD5A activity is responsible for the conversion of active T to the most potent human active androgen DHT (Marchetti et al., 2013). The synthesis of DHT is catalysed by the SRD5A isoforms type 1 (SRD5A1) and type 2 (SRD5A2), with SRD5A1 being expressed in non-genital skin, muscle, adipose tissue and brain, and SRD5A2 being expressed in male reproductive tissue such as prostate and epididymis (Thigpen et al., 1993).

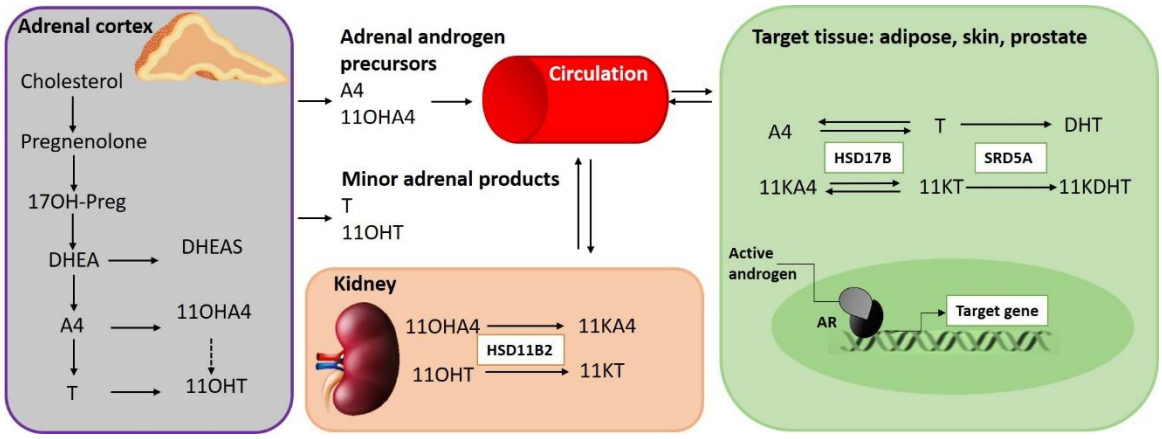

Figure 1.3 Synthesis, circulation and metabolism of classic and 11-oxygenated androgens. Target tissues including adipose tissue, skin and prostate can convert the adrenal androgen precursors A4 and 11KA4 into bioactive androgens via 17β-hydroxysteroid dehydrogenases (HSD17B) enzymes and steroid 5α-reductases (SRD5A). The peripheral conversion of inactive androgen precursors to active androgens by HSD17Bs is a reversible reaction. The bioactive androgens bind the androgen receptor located in the cytoplasm, and the hormone-receptor complex gets translocated to the nucleus, where it acts as transcription factor and modulates the expression androgen-responsive genes. Figure adapted from Turcu et al., 2020.

1.2.6 Regulation of androgen bioavailability by binding proteins in circulation

To avoid an excess of androgen precursors in circulation, the majority of DHEA is sulphated to DHEAS by the PAPS synthase isoform PAPSS2 and sulphotransferase SULT2A1 within zona reticularis of the adrenal gland (Turcu et al., 2015). Despite DHEAS being the most abundant steroid in circulation, this inactive steroid precursor mainly functions as a reservoir for the generation of active androgens. When active androgens are needed, steroid sulphatase (STS) converts DHEAS back to DHEA (Schiffer et al., 2018). Androgen bioavailability for action and metabolism in peripheral tissue is also regulated by the binding of androgens to plasma proteins in circulation. Circulating androgens bind to sex hormone binding globulin (SHBG) and albumin (Hammond, 2016). SHBG has a high affinity for T and DHT in nanomolar ranges and also binds to androgen precursors such as DHEA at a lower affinity (Laurent et al., 2016). Although albumin binds to all steroids with a low affinity, due to its high abundance in circulation it still influences androgen availability (Schiffer et al., 2019). Only unbound steroids are considered 'free' and able to passively diffuse across cell membranes (Mendel, 1989). However, 'free' unbound androgens represent only around 1-2% of circulating androgens, meaning

SHBG and albumin have significant roles in the regulation of availability of androgens in peripheral tissues (Willnow et al., 2010).

1.3 Classic and 11-oxygenated androgens in health and disease

1.3.1 Physiology of circulating androgens

DHEA and DHEAS concentrations peak in early adulthood, followed by an age-related decline, illustrated in **Figure 1.4**. The concentration of circulating T is around ten-fold higher in men than women, due to the major biosynthesis of T in the testes, as opposed to lower production of androgens by the adrenal glands in both sexes and the ovaries in women. Like for DHEA and DHEAS, the concentrations of both classic androgens A4 and T decline with age in both sexes (Eisenhofer et al., 2017). The decrease in classic androgen secretion accompanied with age is due to a multitude of reasons, including the effect of menopause in women, a decrease in androgen biosynthesis in the testes and a decrease in the zona reticularis cell layer and function (Spencer et al, 2007; Golan et al., 2015).

In contrast to classic androgens, the circulating concentrations of 11-oxygenated androgens remain relatively constant throughout the human lifespan **(Figure 1.4)** (Nanba et al., 2019; Davio et al., 2020). 11OHA4 is the second most abundant androgen precursor produced by the human adrenal and has been shown to have higher concentrations than classic androgen precursor A4 in women (Rege et al., 2013; Turcu et al., 2020). In post-menopausal women the concentration of 11OHA4 exceeds classic androgen precursor DHEA (Turcu et al., 2020). In addition, circulating levels of 11KT have been shown to be higher than T in pre-menopausal healthy women (O'Reilly et al.,2017; Rege et al., 2018; Skiba et al., 2019).

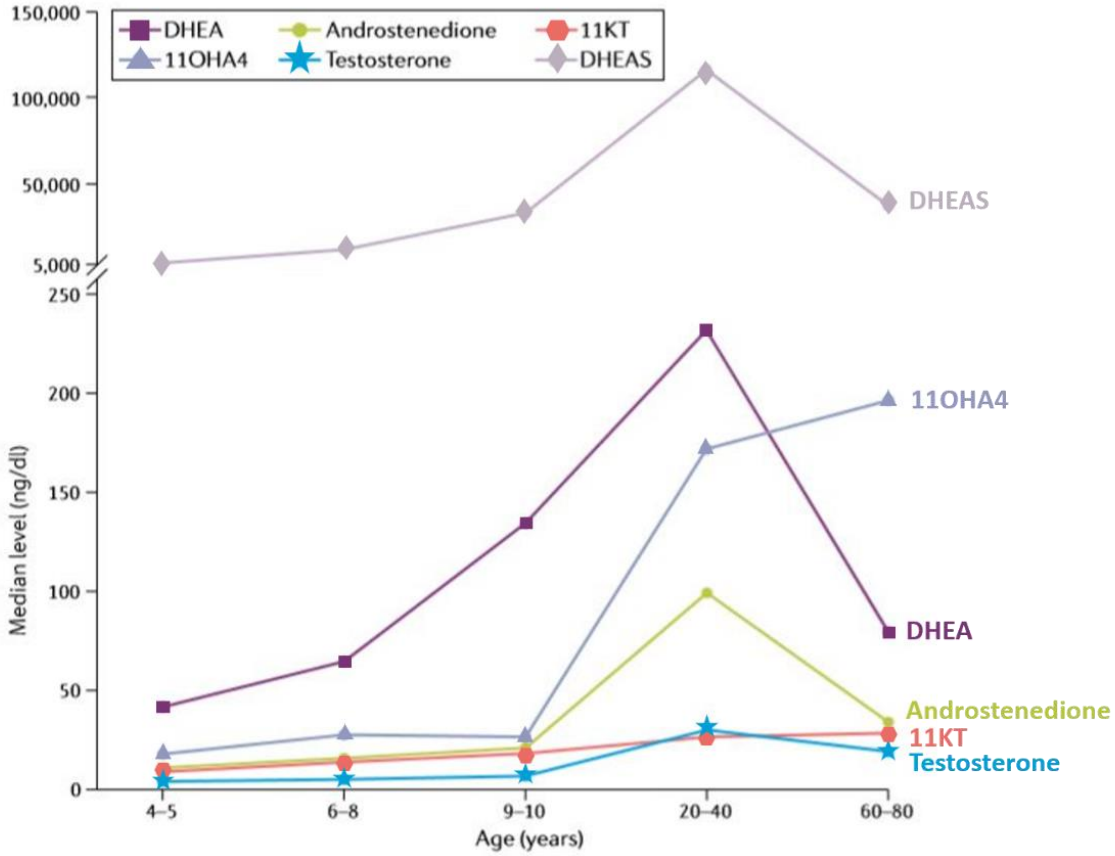

Figure 1.4 The level of key classic and 11-oxygenated androgens and precursors throughout the female lifespan. The most abundant adrenal androgen precursors are DHEA and its sulphate DHEAS, followed by 11OHA4. There is an age-related decline in classic androgens (DHEA, DHEAS, androstenedione, testosterone), which is not observed for 11-oxygenated androgens (11OHA4, 11KT). Figure adapted from Turcu et al., 2020, data (medians) from Rege et al., 2018 and Nanba et al., 2019. Total n = 283 girls and women (aged 4–5 years, n = 22; aged 6–8 years, n = 38; aged 9–10 years, n = 23; aged 20–40 years, n = 100; aged 60–80 years, n = 100).

1.3.2 Androgen excess conditions

Androgen excess is associated with several disorders seen in humans, including congenital adrenal hyperplasia (CAH), polycystic ovary syndrome (PCOS) and castration resistant prostate cancer (Barnard et al., 2019; Turcu et al., 2020).

Congenital adrenal hyperplasia is a group of autosomal recessive conditions due to mutations in genes encoding enzymes essential in adrenal glucocorticoid biosynthesis (Krone et al., 2009). Such genetic defects result in complete or partial enzymatic dysfunction, resulting in a spectrum of clinical symptoms. In all five forms of CAH, steroid precursors accumulate due to the defect in a downstream enzyme and shunt into the pathway with normal functioning enzymes, leading to glucocorticoid deficiency while adrenal androgens and mineralocorticoids might be impaired or upregulated, depending on the enzyme affected (Krone et al., 2009; Pretorius et al., 2017). 21-hydroxylase deficiency accounts for around 95% of all CAH cases and is due to a mutation disrupting the function of the CYP21A2 enzyme (Miller, 2018). CYP21A2 is vital for the mineralocorticoid and glucocorticoid biosynthesis pathways, where it converts progesterone to 11-deoxycorticosterone and 17OHP to 11-deoxycortisol, respectively **(Figure 1.1)** (Turcu et al., 2015). Due to the disruption of CYP21A1 activity, there is an excessive accumulation of 17OHP, and as CYP17A1 is functioning as normal, excessive amounts of 17OHP are converted to A4. Therefore, patients with 21-hydroxylase deficiency have androgen excess, which in severe cases can lead to severe genital virilisation of female new-borns and in milder cases hirsutism and oligomenorrhoea (Speiser et al., 2018). Of interest, it has been shown that 11-oxygenated androgens are the predominant androgens in 21-hydroxylase deficiency and are a more accurate biomarker of adrenal androgen excess than classic androgens in affected patients (Kamrath et al., 2018; Bacila et al., 2019; Turcu et al., 2017). Importantly, Turcu et al (2016) found that levels of 11-oxygenated androgens, including 11OHA4, 11KA4 and 11KT were significantly higher in patients of both sexes with classic 21-hydroxylase deficiency compared to age- and sex-matched controls.

1.3.3 Polycystic Ovary syndrome

Polycystic ovary syndrome is the most common endocrine disorder in women (Wolf et al., 2018). The National Institute of Health (NIH) criteria defines PCOS as a combination of ovulatory dysfunction, hyperandrogenism and polycystic ovarian morphologic features. According to the NIH criteria, PCOS affects around 10% of women worldwide, however, when using the Rotterdam criteria, in which women can display any two of the three features, the incidence may be increased two-fold (Tehrani 2011; Yildiz et al., 2012).

Androgen excess is a defining feature of PCOS and is present in most patients. Hyperandrogenism occurs in around 60-80% of females with PCOS and presents with both phenotypical signs such as hirsutism, male pattern baldness, acne or androgenic alopecia and biochemical signs such as elevated serum androgen levels (McCarney 2016; Balen et al., 2016). The ovulatory dysfunction seen in PCOS can present as absent (amenorrhea) or irregular (oligomenorrhea) menstrual periods (Legro, 2013). Amenorrhea and oligomenorrhea indicate anovulation, and prolonged periods of anovulation are associated with an increased incidence of infertility and difficulty conceiving (Yildiz et al., 2012; Balen et al., 2016). However, the consequences of PCOS are not limited to reproduction only. Women with PCOS have a two-fold increased risk of developing cardiometabolic disease, with women at risk for the development of both metabolic (obesity, insulin resistance, type II diabetes mellitus) and cardiovascular disease (Balen et al., 2016; Yildiz et al., 2012). Many women with PCOS exhibit insulin resistance and as a result compensatory hyperinsulinemia (Baptiste et al., 2010). Of importance, women that only present with symptoms of ovulatory dysfunction and polycystic ovaries without hyperandrogenism have a lower metabolic risk (McCartney et al., 2016). Therefore, androgens have been proposed to be the most relevant and best biomarker to identify the PCOS patients with the highest risk for metabolic complications (Azziz et al., 2006; O'Reilly et al., 2014).

The androgen excess seen in PCOS is due to abnormalities in both adrenal and ovarian steroidogenesis, as well as tissue-specific local androgen excess **(Figure 1.5)**. Liquid chromatography-tandem mass

spectrometry (LC-MS/MS) has become the preferred analytical tool to diagnose the biochemical hyperandrogenism seen in PCOS. LC-MS/MS has high sensitivity and specificity which is important for androgen analysis in women, due to their relatively low concentrations of serum T, which is traditionally measured as the biomarker for androgen excess in PCOS (Keevil 2019). Almost 50% of women with PCOS have a predominantly adrenal hyperandrogenism phenotype as indicated by elevated concentrations of DHEAS, which is exclusively produced in the adrenal glands (Carmina et al., 1986) and this is in particular observed in younger women with PCOS (Elhassan et al., 2018).

A recent study using LC-MS/MS analysis of serum androgens showed that both obese and lean women with PCOS had significantly increased concentrations of classic androgens T, A4 and DHEA compared to age- and BMI-matched controls, but also increased levels of the 11-oxygenated androgens 11OHA4, 11KA4 and 11KT (O'Reilly et al., 2017). The observed increases in DHEA and 11-oxygenated androgens, which are exclusively of adrenal origin, are also a clear indication that the adrenal gland makes the major contribution to the androgen excess observed in PCOS. The same study also showed that the levels of 11KT in serum were higher than the levels of T in both PCOS patients and matched control, adding to the growing body of evidence that 11KT might be the most physiologically relevant androgen in women (O'Reilly et al., 2017). Contributing to the theory that androgen excess is causally linked to the highest risk for metabolic complications, it has been shown that a high salivary T/A4 and T/DHT ratio in PCOS patients is associated with an adverse metabolic phenotype including glucose intolerance, insulin resistance, metabolic syndrome, obesity and oligo-/anovulation (Munkzer et al., 2014; Munkzer et al., 2017).

In PCOS, the ovaries also contribute to hyperandrogenism due to the persistent GnRH secretion from the hypothalamus, as opposed to the normal pulsatile rhythm (McCarney, 2016). The rapid GnRH pulses favour LH synthesis and secretion over FSH within the anterior pituitary (Baskind et al., 2016). The increased LH secretion stimulates the theca cells to produce excessive amounts of androgen, and the decrease in FSH secretion along with androgen excess interferes with normal follicular

development, leading to ovulatory dysfunction (Baskind et al., 2016). The disruption in follicular development in PCOS presents as multiple immature follicles or 'cysts' remaining on the ovaries (Legro 2013) and while androgens are required for physiological follicle maturation, androgen excess arrests follicular development at an early stage (Lebbe et al., 2017).

Tissue-specific androgen activation is an important contributor to local androgen excess in PCOS. Adipose tissue is a target of androgen action and it has been shown that androgen excess contributes to an increase in visceral fat (Pasquali et al., 2019). It has been found that PCOS patients have an increase in local androgen generation in adipose tissue (O'Reilly et al., 2017). Importantly, an overexpression of AKR1C3 has been observed in the adipose tissue of both PCOS patients (Wang et al., 2012; O'Reilly et al., 2017) and women with obesity (Quinkler et al., 2004). As AKR1C3 is the only enzyme within adipose tissue capable of converting A4 to T, this enzyme drives the tissue-specific generation of active androgens leading to local androgen excess (Quinkler et al., 2004). O'Reilly et al (2017) also found that insulin upregulates AKR1C3 expression and activity in human subcutaneous adipocytes of women with PCOS and in the human preadipocyte SGBS cell line.

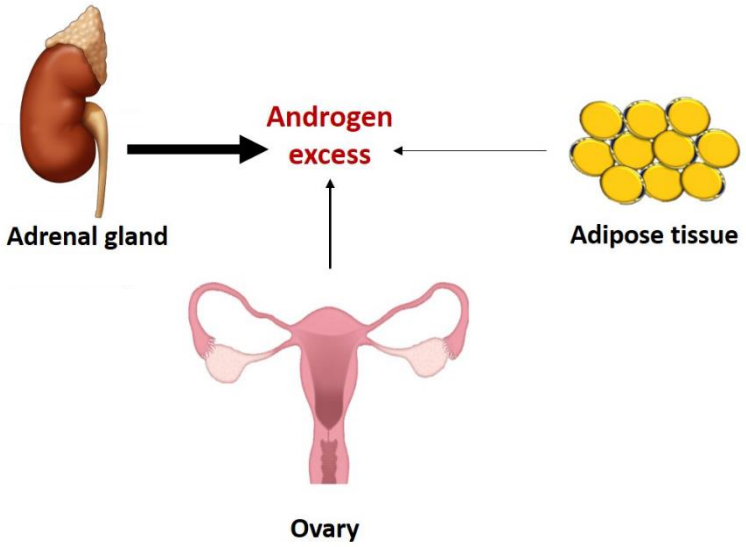

Figure 1.5 The adrenal glands make the major contribution to androgen excess observed in PCOS. The ovaries and tissue-specific androgen activation are also important contributors. Original figure.

1.4 Peripheral blood mononuclear cells

1.4.1 Subsets of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) are blood cells with a round nuclei which encompass a heterogenous cell population of lymphocytes (T cells, B cells and Natural Killer (NK) cells), monocytes and dendritic cells (Kleiveland., 2015). These blood cells are critical components of both the innate and adaptive immune system, vital for defending the body against viral, bacterial and parasitic infection, as well as destroying tumours and foreign substances (Orkin., 2000; Kleiveland., 2015). PBMCs arise from haematopoietic stem cells through the process of haematopoiesis, shown in **Figure 1.6**. In adulthood, haematopoiesis occurs mostly in the bone medullary cavity and generates myeloid lineages (erythrocytes, platelets, neutrophils, eosinophils, basophils, monocytes, macrophages and dendritic cells) and lymphoid lineages (T and B lymphocytes, natural killer cells) (Scheiermann et al., 2015; Orkin 2000).

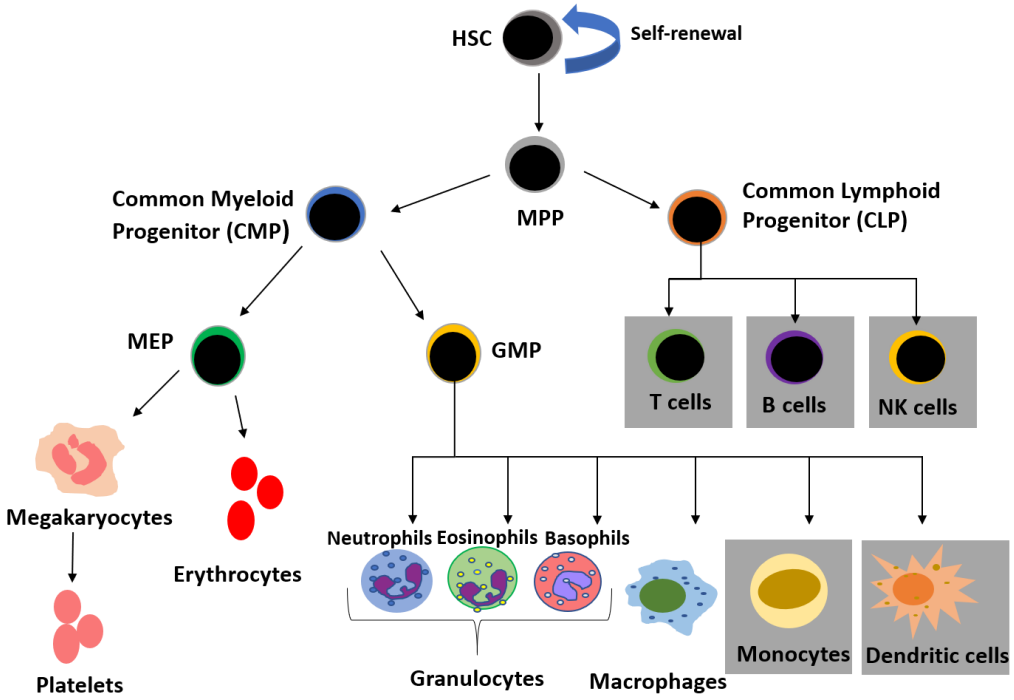

Figure 1.6 Haematopoietic lineages Hematopoietic stem cells possess the ability to self-renew, proliferate and differentiate into all lineages of blood cell. Both the myeloid and lymphoid lineages contain blood cells which make up PBMCs. There is increasing commitment with lineages as cells differentiate. Peripheral blood mononuclear cells are highlighted in grey boxes. Figure adapted from Orkin 2000 and Scheiermann et al., 2015.

The cell frequencies that contribute to the total number of PBMCs in human are shown in **Table 1.1.** The sub-type of PBMC with the highest frequency is lymphocytes (T, B and NK cells). T cells which express the cell surface marker CD3 are the most abundant lymphocyte, with a frequency of 70-85% (Kleiveland 2015). CD3+ T cells can be further classified as CD4+ T helper cells and CD8+ cytotoxic T cells, which have a ratio of 2:1 (Amancha et al., 2019; Kleiveland 2015). Naïve T cells express the T cell receptor and become activated through antigen recognition. Once activated they can elicit a cell-mediated immune response to target antigens within an infected or diseased cell (Kleivelan 2015; Hirahara et al., 2013).

CD19+ B cells make up around 5-10% of the lymphocyte population, they express the B cell receptor and exist as naïve or memory cells waiting for antigen activation. Once activated, B cells can differentiate into plasma cells which, secrete antibodies to target specific circulating antigens.

NK cells are derived from the common lymphoid progenitor. They account for 5-20% of the lymphocyte population and are part of the innate immune system (Kleivelan 2015). NK cells specialise in the killing of virus-infected and malignant cells, they lyse target cells by direct contact with them in the absence of antibody or by antibody dependent cellular cytotoxicity (Bouman et al., 2005). NK cells express $CD56^{+}/CD16^{+}$ or $CD16^{+}/CD16^{+}$ (Bouman et al., 2005).

Monocytes are characterised by the high expression of the CD14 cell surface marker and make up 10-20% of PBMCs. When stimulated, monocytes can differentiate into dendritic cells or macrophages which can then mediate innate immune responses (Qu et al., 2014) Dendritic cells make up 1-2% of the PBMC population. They are antigen presenting cells able to engulf antigens and present fragments of the antigen, which elicits activation of T and B cells (Théry et al., 2001).

Table 1.1: Human PBMC cell types and frequencies. PBMCs comprise of lymphocytes (T cells, B cells and NK cells), monocytes and dendritic cells. Data from Kleivelan, 2015; Scheiermann et al., 2015.

Human Peripheral Blood Mononuclear Cells	% in PBMCs	Function	Markers
Lymphocytes	70-90%		
Total T cells	70-85%		CD3+
CD4+ T cells	25-60% of total CD3 T cells	Coordinate adaptive immunity through activation and regulation of other immune cells	CD3+ CD4+
CD8+ T cells	5-30% of total CD3 T cells	Destroy infected/malignant cells	CD3+ CD8+
Total B cells	5-10%	Secrete antigen-specific antibodies	CD19+
NK cells	5-20%	Trigger apoptosis of virus-infected/malignant cells	CD56+ CD16+
Dendritic Cells	1-2%	Antigen presentation to T cells	
Monocytes	10-20%	Phagocytose foreign objects, antigen presentation	CD14+

1.4.2 Androgen metabolism in PBMCs

Within PBMCs, it has been shown that the conversion of A4 to T by activating, reductive 17β-hydroxysteroid dehydrogenase activity is due to AKR1C3 rather than HSD17B3 (Hammer et al., 2005). The same study found that the inactivation of T to A4 in PBMCs by inactivating, oxidative 17β-hydroxysteroid dehydrogenase activity is due to HSD17B4 (Hammer et al., 2005). In addition, Hammer et al (2005) saw an increase in AKR1C3 activity in the PBMCs from middle-aged men (age range 52-66) when compared to young men (age range 23-29), demonstrated by a higher conversion of A4 to T in the older age group. The production of DHT from T confirmed relevant 5α-reductase activity, with SRD5A1 being the 5α-reductase isoform present in PBMCs.

It has been shown that there is relevant androgen metabolism which occurs in whole blood; Hawley et al (2020) investigated whether the time whole blood was left unseparated influenced serum androgen concentrations after separation, which was analysed by LC-MS/MS. After two hours of whole blood incubation, significant increases in serum 11KT were observed. Additionally, they observed an increase in serum T levels, however, these were less pronounced.

1.4.3 Androgens regulate immune cell function

It has been shown that the AR is expressed in a variety of innate and adaptive immune cells including NK cells, macrophages, monocytes, B cells, and T cells (Shi et al., 2016; Benten et al., 2002; Lai et al., 2009). In addition, the AR is also expressed in hematopoietic stem cells and lymphoid and myeloid progenitor cells (Mierzejewska et al., 2015). Androgens can act directly on immune cells by binding to the AR and influencing the transcription of immune-regulatory genes through DNA-binding-dependent mechanisms (Ben-Batalla et al.,2020). Evidence suggests the androgens have an immunosuppressive role on immune cell types, mostly by reducing the expression pro-inflammatory mediators and promoting anti-inflammatory mediators (Ben-Batalla et al., 2020). Studies have also found androgens are important drivers of the sex dimorphism in infectious and autoimmune diseases, with females being more susceptible to autoimmune disease and less vulnerable to infections than males (Fish et al., 2008; Ngo et al., 2014).

1.4.4 Natural killer cell function and role of androgens

The two major defensive mechanisms that NK cells use to eliminate transformed or virus-infected cells are NK cell cytotoxicity and the secretion of cytokines and chemokines (Hazeldine et al., 2013). It has been indicated that the NK cell stimulatory factor, also named interleukin 12A (IL12A) plays an important role in the activation and mediation of the cytotoxicity of NK cells (Shi et al., 2016). In NK cell cytotoxicity, transformed or virus-infected cells are directly destroyed by granule exocytosis or death receptor ligation (Belizário et al., 2018). Granule exocytosis is the main mechanism by which NK cells offer host protection and is characterised by the secretion of cytotoxic proteins into the immunological synapse between the NK cell and its target (Krzewski et al., 2012). Of note, it has been shown that androgens have an immunosuppressive regulatory role on NK cells, by supressing NK cell proliferation (Page et al., 2006) and decreasing NK cell cytotoxicity (Ben-Batalla et al., 2020). Shi et al (2016) found *in vitro* using human hepatocellular carcinoma cells that the AR could supress NK cell stimulatory factor IL12A at the transcriptional level via direct binding to IL12A promoter region which resulted in repressed efficacy of NK cell cytotoxicity.

1.5 Hypothesis and aims

Androgens are vital in human metabolic physiology and pathophysiology. Both classic and 11-oxygenated androgens contribute to the circulating androgen pool in healthy physiology and in conditions such as PCOS and CAH (Turcu et al., 2020; O'Reilly et al., 2017; Pretorius et al., 2016; Kamrath et al., 2017). Evidence suggests that 11KT is the most physiologically relevant active androgen (Hawley et al., 2019). Tissue-specific androgen activation regulates local androgen signalling but can also contribute to the generation of local androgen excess when dysregulated, as observed in adipose tissue of PCOS patients (Wang et al., 2012). While the pathways and tissue-specificity of the metabolism of classic androgens are well established, pathways of the peripheral metabolism of 11-oxygenated androgens remain to be elucidated. AKR1C3 is vital for the activation of both classic and 11-oxygenated androgens, has been shown to preferentially activate 11-oxygenated androgens over classic androgens *in vitro* (Barnard et al., 2018) and has been shown to be expressed in PBMCs (Hammer et al., 2005). Therefore, the purpose of this study is to examine the metabolism of classic and 11-oxygneated androgens *ex vivo* in PBMCs.

Hypothesis

AKR1C3 in PBMCs preferentially generates the active 11-oxygenated androgen 11KT from its precursors 11KA4 compared to the classic androgen T from its precursor A4, making 11KT the primary active androgen acting in PBMCs.

Aims

- To establish which enzymes are responsible for androgen metabolism in PBMCs
- To assess if classic or 11-oxygenated androgens are preferentially activated by AKR1C3 in PBMCs
- To determine which PBMC subpopulations have predominant AKR1C3 and SRD5A1 activity

2. Materials and methods

2.1 The assessment of androgen concentrations in serum samples after separation from whole blood

The collection of blood for the analysis of steroids in human serum was approved by the Science, Technology, Engineering and Mathematics Ethical Review Committee of the University of Birmingham (ERN_17-0494, ERN_17-0494A and ERN_17-0494B). The exclusion criteria were medication (except oral contraceptives) and chronic or acute diseases known to alter steroid biosynthesis or metabolism. Blood collections occurred between 09:00 h and 11:00 h. Participants gave written informed consent before the blood collection.

To assess the stability of androgens in serum left unseparated after the collection of whole blood, blood was collected from 3 male and 3 female volunteers **(Table 2.1**) in BD Vacutainer® SST™ Tubes (gold closure). The tubes were either immediately centrifuged for separation or left unseparated at room temperature in an air-conditioned room for 1, 2, 4, 6 or 24 hours **(Figure 2.1).** After the defined incubation time samples were centrifuged (2000g, 10 minutes) and the serum was collected and stored at -80 °C until analysis by liquid chromatography tandem mass spectrometry LC-MS/MS.

Table 2.1: Characteristics of the blood donors for the assessment the stability of androgens in serum left unseparated after the collection of whole blood. n/a if information was withheld.

Sex	***Age at collection (years)***	***Body Mass Index (BMI, kg/m^2)***
Male	35	31.1
Male	50	28.8
Male	41	26.5
Female	28	20.7
Female	36	24.0
Female	33	n/a

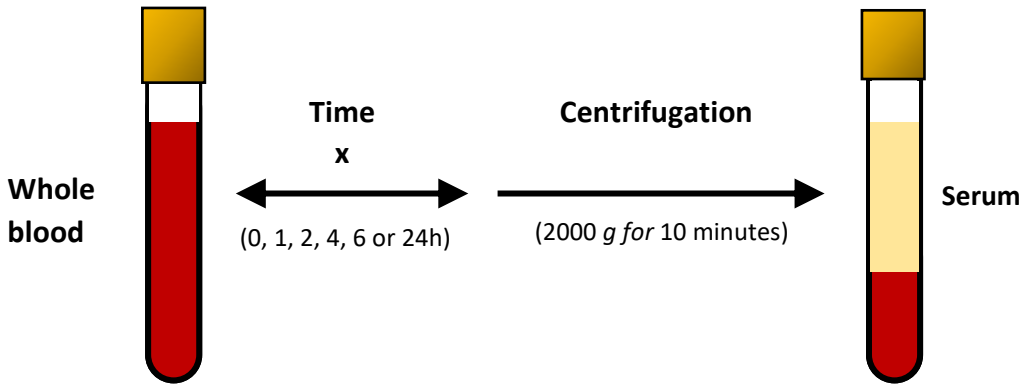

Figure 2.1 Schematic of the experiment assessing the stability of steroids in whole blood. Whole blood was left unseparated for a specified incubation time (0, 1, 2, 4, 6 or 24h), then centrifuged at 2000 *g* for 10 minutes for the separation of serum. Serum samples were stored at -80 °C until steroid quantification by LC-MS/MS. Original figure.

2.2 Isolation of Peripheral Blood Mononuclear Cells

After venous puncture, 50 mL of venous blood were drawn from volunteers and collected in BD Vacutainer™ Plastic K2 EDTA Tubes (lavender closure) for immediate PBMC isolation.

Table 2.2: Characteristics ranges of the blood donors for PBMC isolation for *ex vivo* steroid conversion assays and gene expression.

n-number	*Sex*	*Age at collection range*	*BMI range (kg/m^2)*
Optimisation of the ex vivo steroid conversion assays			
n=3	male	22-28	21.7-23.0
Characterisation of ex vivo steroid conversion and gene expression in male PBMCs			
n=8-12	male	22-72	20.2-30.4
Effect of age on ex vivo steroid conversion and gene expression in male PBMCs			
n=4-5	male	22-30	20.2-29.1
n=4-7	male	53-72	21.2-30.4
Effect of insulin on ex vivo steroid conversion in female PBMCs			
n=3	female	22-32	20.1-22.8

PBMCs were isolated by density gradient centrifugation using Ficoll-Paque Plus (Density 1.077 ± 0.001 g/mL, GE Healthcare). This process is illustrated in **Figure 2.2**. Following the collection of venous blood, the collection tubes were gently inverted. The blood was transferred into 50 mL f using a serological pipette and the EDTA tubes were rinsed with 5 mL of RPMI-1640 (Sigma-Aldrich) supplemented with 100 U/mL penicillin and 0.1 mg/mL streptomycin (RPMI-1640 + antibiotics) to collect and transfer any residual blood. The blood was subsequently diluted with an equal amount of RPMI-1640 + antibiotics. 10 mL of Ficoll-Paque were added to a new 50 mL Greiner Bio-One centrifuge conical-base tubes. The diluted blood was carefully layered on top of the Ficoll-Paque using a serological pipette, ensuring the two layers remained separate, with a final Ficoll-Paque to diluted blood ratio of 1:3. Immediately after the preparation of the gradients, the Greiner Bio-One centrifuge conical-base tubes were centrifuged for 30 minutes in a swinging-bucket rotor at 300*g* without break.

Figure 2.3 illustrates the distribution of blood, Ficoll-Paque and PBMCs in the tubes before and after centrifugation. Using a sterile Pasteur pipette, the PBMC layer was transferred into a new Greiner Bio-One centrifuge conical-base tube. 10 mL of RPMI-1640 + antibiotics were added and the PBMCs were pelleted by centrifugation for 6 minutes at 300*g*. The supernatant was removed, and the cell pellet was suspended in 10 mL of RPMI-1640 + antibiotics. Cell viability was checked using trypan blue exclusion and live cells were counted using a light microscope at x10 magnification. PBMCs were diluted in RPMI-1640 + antibiotics to a cell density of $6x10^6$ cells/mL for the subsequent steroid conversion assay. Additionally, $3x10^6$ PBMCs were harvested by centrifugation and the cell pellet was suspended in 400 µL of TRI Reagent® (Sigma Aldrich) and stored at -80°C for subsequent quantitative PCR analysis.

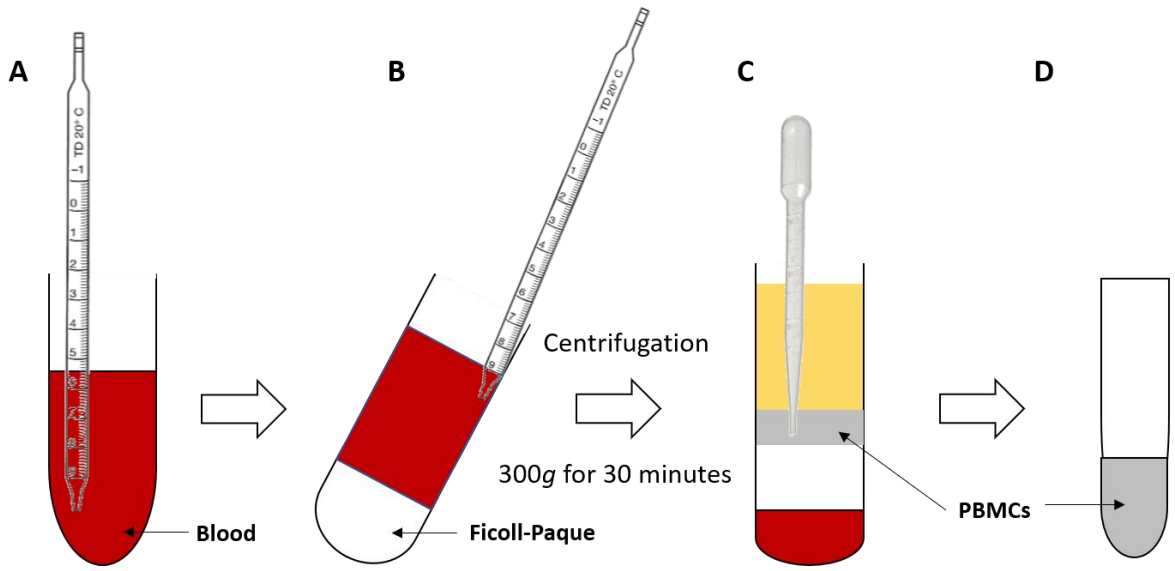

Figure 2.2 Process of PBMC isolation from whole blood by density gradient centrifugation using Ficoll-Paque. (A) An equal amount of RPMI-1640 is added to the blood sample. **(B)** A Greiner Bio-One centrifuge conical-base tube containing Ficoll-Paque is held horizontal and the diluted blood is slowly added to the side of the tube to create two distinct layers of blood and Ficoll-Paque. **(C)** After centrifugation, PBMCs are situated in the layer above the Ficoll-Paque. **(D)** PBMCs are transferred into a new conical tube. Original figure.

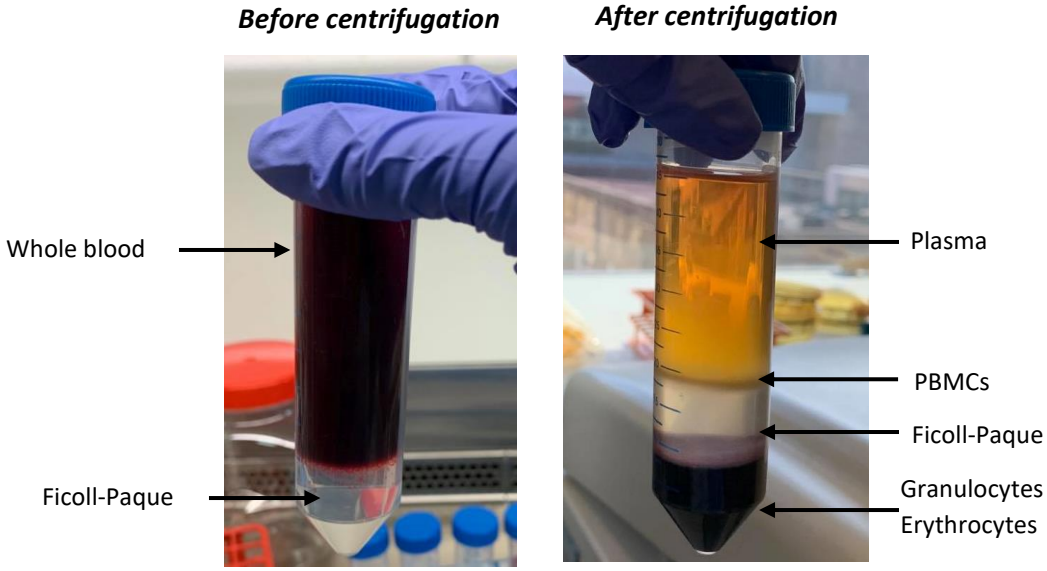

Figure 2.3 Whole blood and Ficoll-Paque before and after centrifugation. Original photo.

2.3 Natural killer cell isolation

A leukocyte cone from an anonymous donor was obtained from NHS Blood and Transplant Birmingham. The use of leukocyte cones from NHS Blood and Transplant for research was approved by the Science, Technology, Engineering and Mathematics Ethical Review Committee of the University of Birmingham (ERN_14-0446). PBMCs were isolated following the protocol described in section 2.2. NK cells were isolated from the PBMCs using the Magnetic Cell Separation MACS® human NK cell isolation kit (Miltenyi Biotec) in accordance with the manufacturer's protocol. An overview of the principles of magnetic cell separation of NK cells is shown in **Figure 2.4**. NK cells viability was checked by trypan blue exclusion. Live cells were counted and diluted in RPMI-1640 + antibiotics to a cell density of $6x10^6$ cells/mL for the subsequent steroid conversion assay. Additionally, NK cell pellets of $9x10^6$ cells were suspended in 400 µL of TRI Reagent® (Sigma Aldrich) and stored at -80°C for subsequent quantitative PCR analysis.

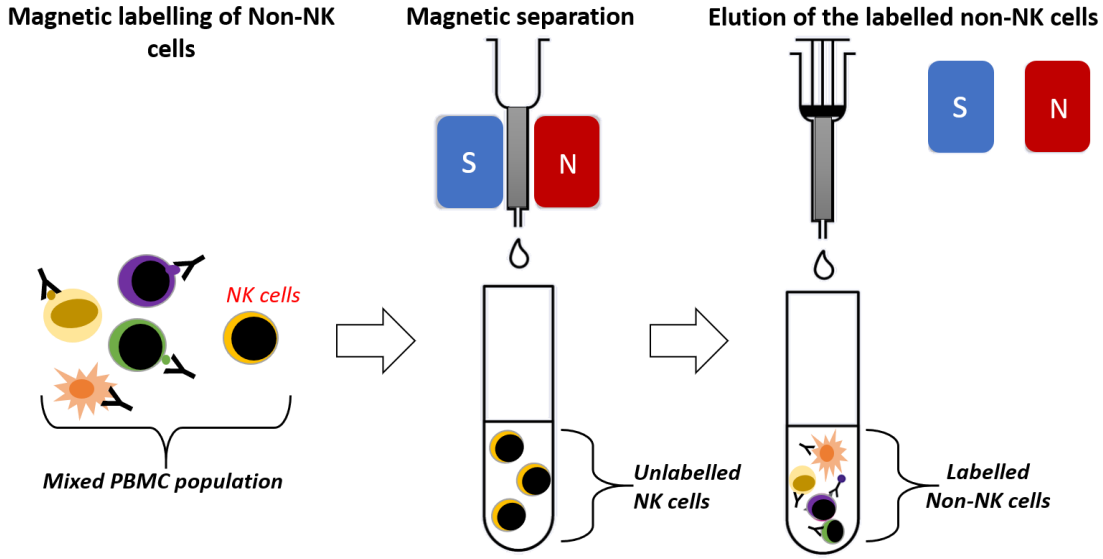

Figure 2.4 Principles NK isolation by negative selection using magnetic cell separation.

A cocktail of biotin-conjugated antibodies (CD3+/CD14+/CD19+) are used to bind to cell surface antigens on non-NK cells in the PBMC population (T cells, monocytes, B cells, dendritic cells), which are subsequently magnetically labelled, while NK cells remain unlabelled. The cell solution is passed through a column with ferromagnetic spheres in a, which amplify the magnetic field generated by the MACS® separator. During separation, the magnetically labelled non-NK cells are retained by ferromagnetic spheres within the column, whilst the unlabelled NK cells can freely flow through and be collected. After a washing step, the column is removed from the magnetic field of the MACS®separator and the magnetically labelled non-NK cells are eluted from the column using a syringe. Figure adapted from Miltenyi Biotec.

2.4 Ex vivo steroid conversion assay

$3x10^6$ PBMCs or NK cells in a final volume of 500 µL RPMI-1640 + antibiotics in 1.5 mL Eppendorf tubes were spiked with a final concentration of 100 nM of one of the following steroids from stock in methanol: DHEA, A4, T, 11KA4, 11KT, 11OHA4 or 11OHT. The final methanol concentration in the incubation was 0.00304%. For each experiment, incubations of cells spiked with methanol only, and cell-free incubations for each substrate tested were prepared. In addition, medium spiked with 100 nM of the respective steroid was prepared and immediately frozen at -20°C until LC-MS/MS analysis. Technical replicates with cells from the same donor were prepared if enough cells were available. Cells were incubated for 2, 4, 6 or 24 h at 37 °C in a hybrid oven to allow for continuous agitation during the incubation. At the end of the incubation period, the tubes were centrifuged for 2 minutes at 10,000 rpm. The supernatant was stored at -20 °C for steroid analysis by LC-MS/MS. The cell pellet was washed in phosphate buffered saline, suspended in 100 µL RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1 mM DTE, 0.1 mM PMSF, 0.1 mM EDTA) and stored at -80 °C for subsequent protein quantification. Total protein content was determined in the supernatant after cell lysis using the DC Protein Assay (Bio-Rad).

2.5 Steroid conversion assay with insulin treatment

PBMCs were isolated from the blood of 4 women **(Table 2.2).** $3x10^6$ PBMCs in a final volume of 500 µL RPMI-1640 + antibiotics in 1.5-mL Eppendorf tubes were treated with 10 nM Insulin (Sigma-Aldrich) or vehicle and incubated for 4 hours in a hybrid oven at 37°C. After 4 hours, paired insulin-treated and non-treated PBMCs were spiked with the relevant steroid substrates.

2.6 Steroid quantification by liquid chromatography-tandem mass spectrometry

2.6.1 Principles of liquid chromatography-tandem mass spectrometry

Ultra high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) is regarded the most accurate method for quantifying steroids and has assisted in the profiling of classic and 11-oxygenated androgens in serum, urine and saliva (Storbeck et al., 2018; Schiffer et al., 2019). LC-

MS/MS offers specific and sensitive high-throughput analysis and can be used for the simultaneous quantification or “multiplexing” of several analytes in one analytical run (Taylor et al., 2015). An advantage of LC-MS/MS is that multiple related steroids can be analysed without the cross-reactivity that occurs in some immunoassays which can cause inaccurate results (Storbeck et al., 2018; Wudy et al., 2018). LC-MS/MS combines the separation abilities of ultrahigh-performance liquid chromatography (UHPLC), and the mass analysis abilities of mass spectrometry to provide sensitive and specific detection and quantification of analytes.

Firstly, the mixture of analytes extracted from a biological sample is chromatographically separated by UHPLC. Reversed-phase chromatography is the separation technique most commonly used for steroid analysis and separation is achieved due to the differential interactions of analytes with the nonpolar stationary phase and polar mobile phase, which is dependent the polarity of a compound (Taylor et al., 2015). The chromatographic retention time is defined as the time taken for the analyte to pass through the stationary column (Dunn et al., 2019). After passing the chromatography column, analytes reach the ion source of the mass spectrometer **(Figure 2.5)**. Electrospray ionisation is a common ionisation technique used for steroid analysis and was used in this project. In the electrospray ionisation source, the solvent is removed and the compounds are ionised (Ho et al., 2003). Triple quadrupole tandem mass spectrometers are used for steroid quantification: The ions pass through a first quadrupole mass filter, which only allows ions of a selected mass to charge ratio (m/z) to pass into the second quadrupole or collision chamber (Adaway et al., 2015). This selected ion is named ‘parent ion’. In the collision chamber a gas such as argon is present. Kinetic energy is applied to this gas causing it to collide with the parent ions leading to their fragmentation (Adaway et al., 2015). A third quadrupole selects specific fragment ions, known as the ‘product ions’, and allows them to pass to the detector. The specific pair of m/z values of the parent and its product ion is known as a ‘transition’ and can be written as parent m/z > product m/z. For example, the protonated molecular parent ion of testosterone $(M+H)^+$ has a m/z of 289. When it fragments in the collision cell, one of the dominant ion fragments has a m/z of 97. Therefore, this transition would be

referred to as m/z 289 > 97 (Hawley et al., 2019). Multiple transitions can be monitored in the mass spectrometer in succession, allowing for multiplexing of several analytes and their internal standard. This is known as multiple reaction monitoring (Adaway et al., 2015).

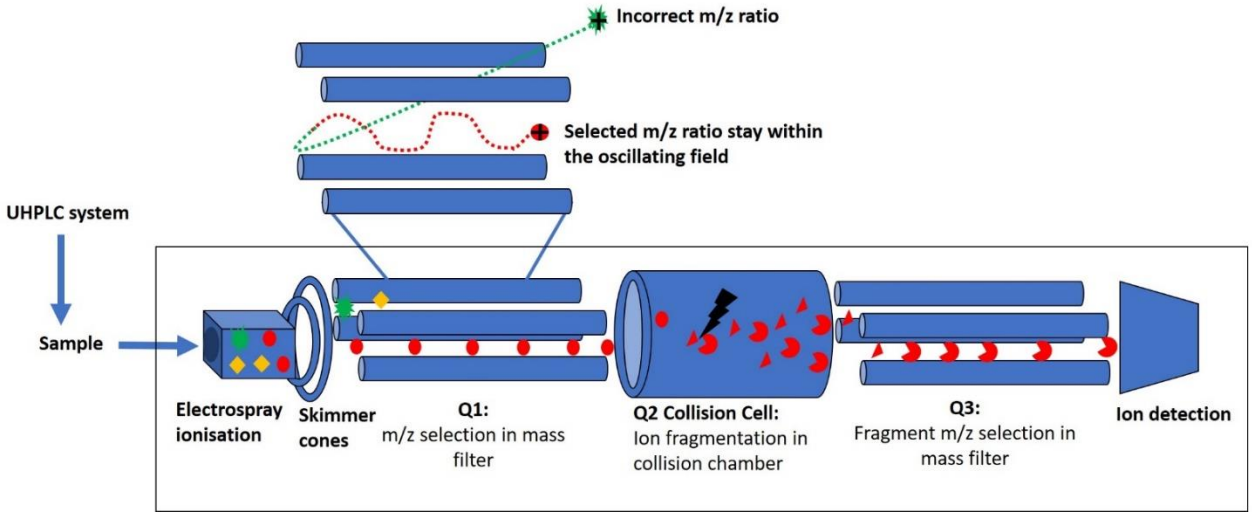

Figure 2.5 Principles of LC-MS/MS.

UHPLC system: Analytes are chromatographically separated.
***Electrospray ionisation*:** The analytes separated by UHPLC are ionised
Skimmer Cones: Preferentially sample gas phase ions and reduce the gas load entering
Q1 Mass filter: Filter ions according to their m/z as they pass through the central axis of four parallel rods of equal distance
Q2 Collison chamber: Ions that emerge from Q1 collide with gas molecules such as argon, which causes the analyte to fragment
Q3 Mass filter: Filters fragment ions after collision, allowing for a specific identification of the original molecule
Ion detection: Ions are detected and transformed into a signal
Figure adapted from Adaway et al., 2015.

2.6.2 Calibration series and internal standard preparation for LC-MS/MS

Standards for analytes were purchased as powders (**Table 2.3**). Stock solutions at 1 mg/mL for each analyte were prepared in methanol (Biosolve) and stored at -80 °C. The stock solutions were used to prepare a joint stock solution in methanol containing all 12 analytes listed in **Table 2.3** at 1000 ng/mL or 1 ng/mL. Using the joint stock solutions, calibrators at 12 different concentrations (**Table 2.4**) were prepared by spiking RPMI-1640 medium. Calibrators were stored at -20°C. An internal standard (IS) mix containing the following stable isotopically labelled steroids at final concentrations of 1 µg/mL was prepared in methanol and stored at -20°C: DHEA-d6, A4-d7, Test-d3, DHT-d3, Adiol-d3, 11KT-d3, 11OHA4-d7. If an analyte did not have its own IS, the most similar IS with regards to polarity and retention time was used for the generation of calibration curves and quantification.

Table 2.3: Steroid measured by LC-MS/MS with the suppliers of their standards and the stable isotope labelled internal standard used in quantification.

Steroid abbreviation	Steroid trivial name	Supplier of standard and catalogue #	Internal standard for quantification
DHEA	Dehydroepiandrosterone	Sigma Aldrich D4000	DHEA-d6
A4	Androstenedione	Sigma Aldrich 46033	A4-d7
5α-dione	5α-androstenedione	Steraloids A2280	DHT-d3
T	Testosterone	Sigma Aldrich T6147	Test-d3
DHT	5α-dihydrotestosterone	Sigma Aldrich A8380	DHT-d3
11OHA4	11-hydroxyandrostenedione	Sigma Aldrich A3009	11OHA4-d7
11OH-5adione	11-hydroxy-5α-androstenedione	Steraloids A8950	11OHA4-d7
11KA4	11-ketoandrostenedione	Sigma Aldrich 284998	11KT-d3
11KT	11-ketotestosterone	Steraloids A6720	11KT-d3
11OHT	11-hydroxytestosterone	Steraloids A5760	11OHA4-d7
An	Androsterone	Steraloids A2420	An-d4
Adiol	Androstenediol	Sigma Aldrich A7755	Adiol-d3

Table 2.4: Concentrations of the calibration series for each analyte in ng/mL and approximate concentration in nM calculated for a molecular weight of 300.

Name	Concentration (ng/mL)	Approximate concentration (nM)
C0	0	0
C1	0.01	0.03
C2	0.05	0.17
C3	0.1	0.33
C4	0.25	0.83
C5	0.5	1.67
C6	1	3.33
C7	5	16.67
C8	10	33.33
C9	25	83.33
C10	50	166.67
C11	100	333.33

2.6.3 *Sample preparation for LC-MS/MS*

Medium samples from incubations with PBMCs and serum samples were extracted using liquid-liquid extraction. 400 µL of each sample and calibrator were pipetted into dimethyldichlorosilane treated glass tubes. 10 µL of the IS mix were added to each sample and the samples were briefly mixed by vortexing. 2 mL of methyl tert-butyl ether were added to each sample. Samples were vortexed for 10 minutes at 1500 rpm. Following this, the samples were incubated at room temperature for 40 minutes for phase separation. Using a glass Pasteur pipette, the organic phase was transferred into 700-µL glass tube inserts (Randox) in a 2-mL square well 96-well plate (Porvair, Leatherhead, UK) and was evaporated under a nitrogen stream, sealed and stored at -20°C until analysis. Samples were reconstituted in 200 µL of 50% (v/v) methanol in water, vortexed at 600 rpm for 2 minutes and centrifuged at 1200*g* for 5 minutes.

2.6.4 Ultra high-performance liquid chromatography

Steroids were quantified by LC-MS/MS as previously described (O'Reilly et al., 2017). Chromatography was performed using an ACQUITY ultra-performance liquid chromatography system (Waters, Manchester, UK), and an ACQUITY HSS T3 column (2.1 mm × 50 mm, 1.8 µm; Waters) at 60 °C. 20 µL of the reconstituted samples were injected. Mobile phase A consisted of 0.1% formic acid in UHPLC

grade water (Fisher chemical), mobile phase B of 0.1% formic acid in UHPLC grade methanol (Biosolve). A linear gradient from 45% to 75% of mobile phase B over five minutes was applied at a flow rate of 0.6 mL min^{-1} to separate the analytes. The autosampler was maintained at 10°C.

2.6.5 Tandem mass spectrometry

Following chromatographic separation, the LC eluate was injected into a XEVO^{TM} TQ-XS tandem mass spectrometer (Waters, Manchester, UK), which was operated in positive electrospray ionisation mode. The capillary voltage was maintained at 1.5 kV and the source temperature was 150°C. The desolvation temperature and gas flow were 600°C and 1200 L/h, respectively. The cone gas was 150 L/h. All transitions (**Table 2.5**) were monitored in multiple reaction monitoring mode. MassLynx software version 4.2 (Waters) was used for system control and TargetLynx software was used to process and quantify data. **Figure 2.6** shows an example chromatogram for analytes A4 and T after an LC-MS/MS run. The ratios of the peak areas of analyte and internal standard were plotted against the nominal concentrations of the calibrators. A 1/x weighting and linear least square regression were used to produce the calibration curves. The assay performance was validated against published criteria's including the Food and Drug Administration's (FDA) published guidelines.

Table 2.5: m/z transitions of the multiple reaction monitoring for target analytes and internal standards.

Analyte	Retention time (min)	Mass transition (*m/z*) Parent > Product Quantifier Qualifier
DHEA	2.61	271 > 253 289 > 271
A4	2.18	287.1 > 97.0 287.1 > 108.9
5αdione	2.92	289.2 > 253.1 289.2 > 213.1
T	2.43	289.1 > 96.9 289.1 > 109.0
DHT	3.10	291.3 > 81.0 291.1 > 255.2
11OHA4	1.45	303.1 > 285.09 303.1 > 267.1
11OH-5αdione	1.76	
11KA4	1.14	301.0 > 121.0 301.0 > 265.2
11KT	1.28	303.1 > 121.0 303.1 > 259.1
11OHT	1.59	305.1 > 269.1 305.1 > 121.0
An	3.76	291.1 > 273.2 291.1 > 255.2
Adiol	3.61	275.3 > 257.2 275.3 > 81.0
Internal standard	**Retention time (min)**	**Mass transition (*m/z*)**
DHEA-d6	2.59	295.2 > 277.1
A4-d7	2.16	294.1 > 96.9
DHT-d3	3.09	294.1 > 258.1
T-d3	2.41	292.1 > 96.9
11OHA4-d7	1.42	310.1 > 292.1
11KT-d3	1.28	306.4 > 262.1
An-d4	3.61	302.2 >265.9
Adiol-d3	3	284.3 > 81.0

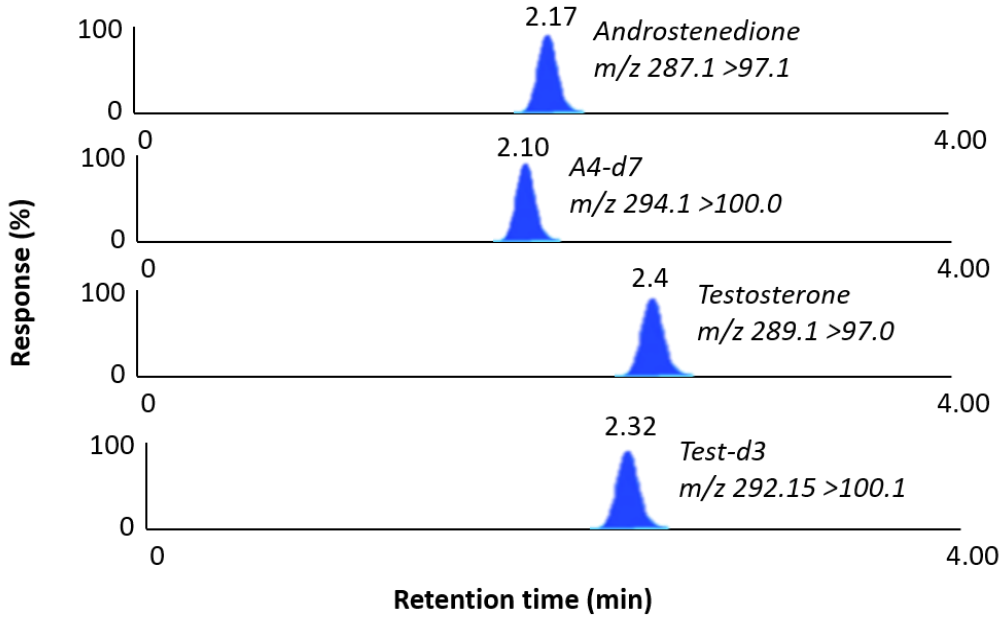

Figure 2.6 Sample LC-MS/MS chromatograms for testosterone and androstenedione, along with their internal standards. Sample retention times and m/z transitions are shown. Original figure, sample m/z transitions from Schiffer et al., 2019.

2.6.6 Ultra-high performance supercritical fluid chromatography-tandem mass spectrometry

11-keto-5α-dihydrotestosterone (11KDHT) and 11-keto-5α-androstenedione (11K-5α-dione) were analysed by ultra-high performance supercritical fluid chromatography-tandem mass spectrometry as previously described (Quanson et al., 2016).

2.6.7 Data and statistical analysis

The steroid concentrations determined in the samples from PBMC incubations were normalised for the total protein content of the incubation. If the concentration of a sample was below the LLOQ, this was represented as 0 nM. The data was analysed and visualised using GraphPad Prism 8.4.3 software. For the serum steroid levels, statistical analysis was performed on the concentrations (nM) using ANOVA followed by Dunnett's Multiple Comparison test to compare each time point against 0 hours. For the ex vivo steroid conversion assay, statistical analysis was performed using the Wilcoxon matched-pairs signed rank test or Mann-Whitney test as appropriate.

2.7 Gene expression analysis by quantitative PCR

2.7.1 Principles of quantitative PCR

The Polymerase Chain Reaction (PCR) is a technique used to amplify DNA which allows for a detectable signal for quantification. Firstly, denaturation occurs in which the temperature is raised to 90-95°C to allow all double-stranded DNA to separate and form single-stranded DNA (Lornez, 2012). During annealing, the temperature is cooled to 40-60 °C which allows primers to attach to the target single-stranded DNA (Lorenz, 2012). During the extension, the temperature is raised to 70-75°C and the DNA polymerase extends the DNA from the primers, to create a new double-stranded DNA (Lorenz, 2012). A thermal cycler allows for an automated repetition of heating and cooling for ~35 cycles in order to amplify the DNA.

Quantitative PCR (qPCR) is an advanced form of the Polymerase Chain Reaction, in which the process of amplification is monitored in real-time and relies on the generation of fluorescence, which can be detected during each PCR cycle (Arya et al., 2005). A common fluorescent technique is to use a hydrolysis probe-based detection solution, such as TaqMan (**Figure 2.7**) (Arya et al., 2005). TaqMan probes are fluorescently labelled DNA oligonucleotides (Nagy et al., 2017). The 5' end of the probe is labelled with a fluorescent reporter molecule such as FAM and the 3'end of the probe is labelled with a non-fluorescent quencher molecule, which effectively quenches the output from the reporter (Arya et al., 2005; Nagy et al., 2017). When the reporter and quencher are attached, the overall level of fluorescent output is low (Nagy et al., 2017). During annealing, the probe binds downstream of the primer and is subsequently cleaved by the polymerase enzyme (Nagy et al., 2017). Cleavage results in the separation of the reporter and quencher, and as a result the quencher no longer inhibits the reporter meaning the level of fluorescence increases (Arya et al., 2005). With every cycle of PCR, there is an exponential increase in fluorescence (Nagy et al., 2017), which is monitored in real time.

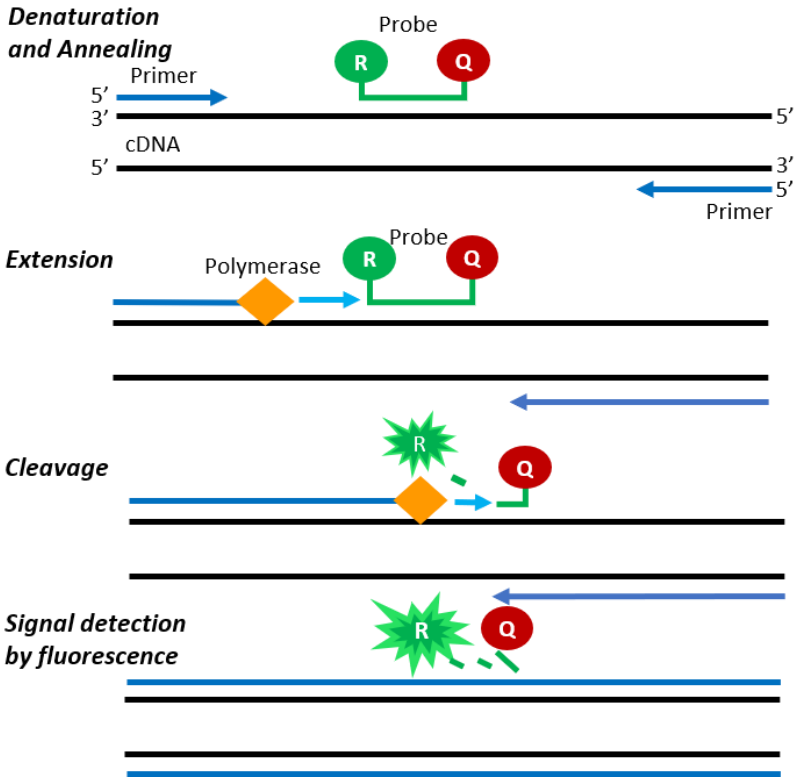

Figure 2.7 Quantitative PCR is monitored in real-time using TaqMan probes to generate fluorescence. Denaturation forms single-strands of cDNA followed by the annealing of primer and TaqMan probe to the single-stranded DNA. During the extension phase, the DNA polymerase synthesises a complimentary DNA strand. The polymerase then cleaves the TaqMan probe which contains a dye label (e.g. FAM) on the 5' end and a non-fluorescent quencher on the 3' end. Cleavage results in the separation of reporter and quencher, which causes fluorescence. **R** Reporter, **Q** Quencher. Information from Arya et al., 2005. Original figure.

2.7.2 RNA extraction, cDNA synthesis and qPCR

Cell lysates stored in TriReagent at -80°C were thawed at room temperature. To extract RNA, chloroform was added to each sample, this was vortexed and incubated at room temperature for 3 minutes. Following this, samples were centrifuged at 13,000 rpm for 20 minutes at 4°C. The aqueous phase was then transferred to a RNAse free Eppendorf and RNA was purified using a single step extraction method (RNeasy Mini Kit, Qiagen) using the manufacturer's protocol. RNA concentrations were determined using a Nanodrop spectrometer from the absorbance of the sample at 260 nm. Reverse transcription of the RNA samples was performed using Applied Biosystems™ TaqMan™ Reverse Transcription Reagents using the manufacturers protocol. A TaqMan™ Gene Expression Assays (FAM-labelled) with the SensiFAST™ Probe Hi-ROX kit (Bioline) was prepared on a 384-well

plate (Thermofisher). Each sample was prepared in duplicate. qPCR was performed using an applied biosystems 7900HT sequence detection system (Perkin Elmer, Applied Biosystems). Data was expressed as threshold cycle (Ct) values. ΔCt was calculated as Ct [Target]-Geometric mean (Ct [HPRT1], Ct [GAPDH]). The mean ΔCt for the duplicates of each sample was calculated. Gene expression in arbitrary units (A.U.) was calculated as 1000*2^-ΔCt. For any targets that were not reproducibly detected in duplicate reactions, relative gene expression is shown as 0. The TaqMan™ Gene Expression Assays used are summarised in **Table 2.6**.

Table 2.6: TaqMan™ Gene Expression Assay used in qPCR.

Gene of interest	TaqMan™ Gene Expression Assay
GAPDH (housekeeping control)	Hs99999905_m1
HPRT1 (housekeeping control)	Hs02800695_m1
AKR1C3	Hs00366267_m1
SRD5A1	Hs00602694_mH
SRD5A2	Hs00165843_m1
HSD17B2	Hs00157993_m1
HSD17B3	Hs00970002_m1
HSD17B4	Hs00264973_m1
HSD11B1	Hs01547870_m1
HSD11B2	Hs00388669_m1

2.8 Analysis of published RNAseq data from PBMC populations

To investigate the expression of *AKR1C3* and *SRD5A1* in different PBMC populations, publicly available RNAseq gene expression data from 15 FACS-sorted PBMC subpopulations including B-cells, T-cell populations and NK cells (Schmiedel et al., 2018; dice-database.org) was analysed. The database contained expression data from 37 female and 54 male healthy donors (age range of 18-61). Expression data in transcript per million (TPM) was downloaded and plotted in GraphPad Prism. Statistical analysis was performed by one-way ANOVA, followed by Tukey's multiple comparisons test.

3. Results

3.*1 AKR1C3 and SRD5A1 are the major androgen-activating enzymes in PBMCs*

qPCR was used to identify the relevant enzymes and isoforms of androgen metabolism in PBMCs and their mRNA expression levels were examined in PBMCs from 14 male donors **(Table 2.2)**. As shown in **Figure 3.1A**, *AKR1C3* showed the highest relative expression (n=14) whilst expression of *HSD17B3*, the other human reductive 17β-hydroxysteroid dehydrogenase, which is generally considered a testis-specific enzyme, was detected at negligible levels compared to *AKR1C3* (detected in 13/14 donors). This indicates that AKR1C3 is the key reductive activating 17β-hydroxysteroid dehydrogenase in male PBMCs. High relative expression of 5α-reductase type 1 (*SRD5A1)* (n=14) suggests it is responsible for 5α-reduction activity seen in subsequent PBMC incubations, whilst 5α-reductase type 2 (*SRD5A2)* was undetectable. 17β-hydroxysteroid dehydrogenase type 2 (*HSD17B2)* was undetectable in sample from all donors. 17β-hydroxysteroid dehydrogenase type 4 (*HSD17B4*) was detected in 6/14 donors, suggesting it is responsible for oxidative inactivating 17β-hydroxysteroid dehydrogenase activity seen in following PBMC incubations. The variable detection of HSD17B4 may be accounted for by the differences in age of donors. However, despite reproducible detection of *HSD17B4* in duplicate samples from the same donor, the relative expression data for *HSD17B4* data is not represented in **Figure 3.1** as it did not pass the quality control due to limited precision. Expression of 11β-hydroxysteroid dehydrogenase type 1 (*HSD11B1*) was detectable at low levels (detected in 13/14 donors), whilst 11β-hydroxysteroid dehydrogenase type 2 (*HSD11B2)* mRNA was not detected. Overall, these results confirm that AKR1C3 and SRD5A1 are the major androgen-activating enzymes in male PBMCs.

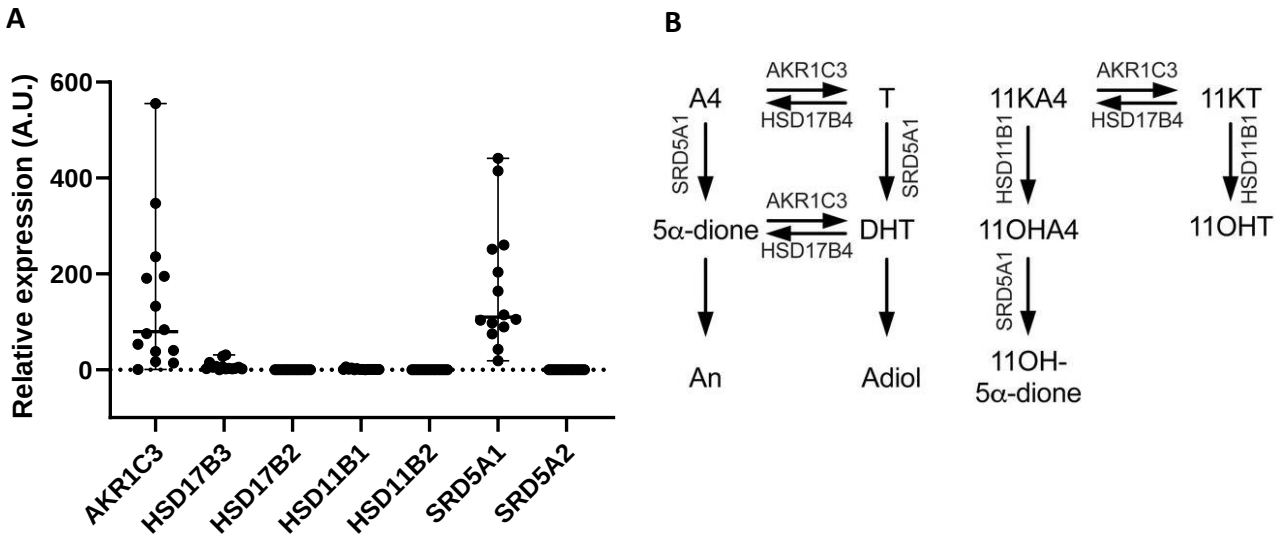

Figure 3.1 Relative expression of genes encoding enzymes of androgen metabolism in PBMCs.

(A) PBMCs were isolated from healthy males (n=14; age range 22-72 years; BMI range 20.2-30.4 kg/m^2). Gene expression was assessed by qPCR and normalised to the expression of the housekeeping genes HPRT1 and GAPDH. Arbitrary units (A.U.) were calculated as 1000*2^-ΔCt. *HSD17B4* was detected in samples from 6/14 donors but is not included as it did not pass the quality control due to limited precision. **(B)** Schematic representation of the steroidogenic enzymes in PBMCs responsible for activation and inactivation of androgens in classic and 11-oxygenated androgen pathways.

3.2 PBMCs metabolise classic and 11-oxygenated androgens ex vivo: Proof-of-principle and optimisation of the incubation time

To identify relevant substrates for androgen metabolism in PBMCs and incubation times for follow-up experiments, *ex vivo* time-dependent experiments with a range of androgen precursors and active androgens from both the classic and 11-oxygenated androgen pathways were performed. PBMCs were isolated from three males **(Table 2.2)** and *ex vivo* steroid conversion experiments were carried out with substrates from the classic androgen pathway (DHEA, A4 and T) and the 11-oxygenated androgen pathway (11KA4, 11KT, 11OHA4 and 11OHT). Incubations were performed for either 2, 6 or 24 hours and product formation was quantified by LC-MS/MS.

As shown in **Figure 3.2**, metabolite formation was linear over 24 hours for all substrates and 24 hour incubations resulted in quantifiable levels of all products. Therefore 24 hour incubations were used for all follow-up experiments. After 24 hours, incubation of PBMCs with A4 primarily yielded 5α-dione as a result of 5α-reductase activity by SRD5A1, followed by down-stream conversion to An. Additionally, activation of A4 to T was observed (see Panel B of **Figure 3.2**). Panel C of **Figure 3.2** shows the prominent conversion of 11KA4 to 11KT due to activating, reductive 17β-hydroxysteroid dehydrogenase activity by AKR1C3. In addition, negligible conversion to 11OHT or 11OHA4 was observed (**Figure 3.2C**). Incubations with T revealed efficient back-conversion to A4 due to inactivating, oxidative 17β-hydroxysteroid dehydrogenase activity (**Figure 3.2D**). Additionally, relevant 5α-reduction of T by SRD5A1 led to the formation of the most potent androgen DHT (**Figure 3.2D**). As shown in **Figure 3.2E**, incubation of PBMCs with 11KT yielded 11OHT as a result of *HSD11B1* activity, and only minor back-conversion to 11KA4 compared to the generation of A4 from T (**Figure 3.2B**). After 24 hours, only low levels of DHEA were converted to A4 and T (**Figure 3.2F**), which signifies only minor 3β-hydroxysteroid dehydrogenase activity in PBMCs and a negligible role of DHEA as substrate for the generation of active androgens in PBMCs. Therefore, DHEA metabolism in PBMCs was not further investigated. Incubation of PBMCs with 11OHT results in the conversion to 11KT, due to HSD11B2 activity (**Figure 3.2G)**. Finally, when 11OHA4 serves as a substrate it becomes 5α-reduced to 11OH-5α-dione, however this reaction is negligible compared to the conversion of the other substrates (**Figure 3.2H**). In the order of the amounts of products quantified, the most relevant substrates for metabolism by PBMCs are: 11KA4, A4, T and 11KT. Only minor metabolism of 11OHA4, 11OHT, and DHEA was observed. Using a previously published ultra-high performance supercritical fluid chromatography-tandem mass spectrometry assay (Quanson et al., 2016) it was found that no 5α-reduced products of the 11-ketoandrogens 11KA4 and 11KT were detected (11-keto-5α-androstenedione and 11-keto-5α-dihydrotestosterone, respectively).

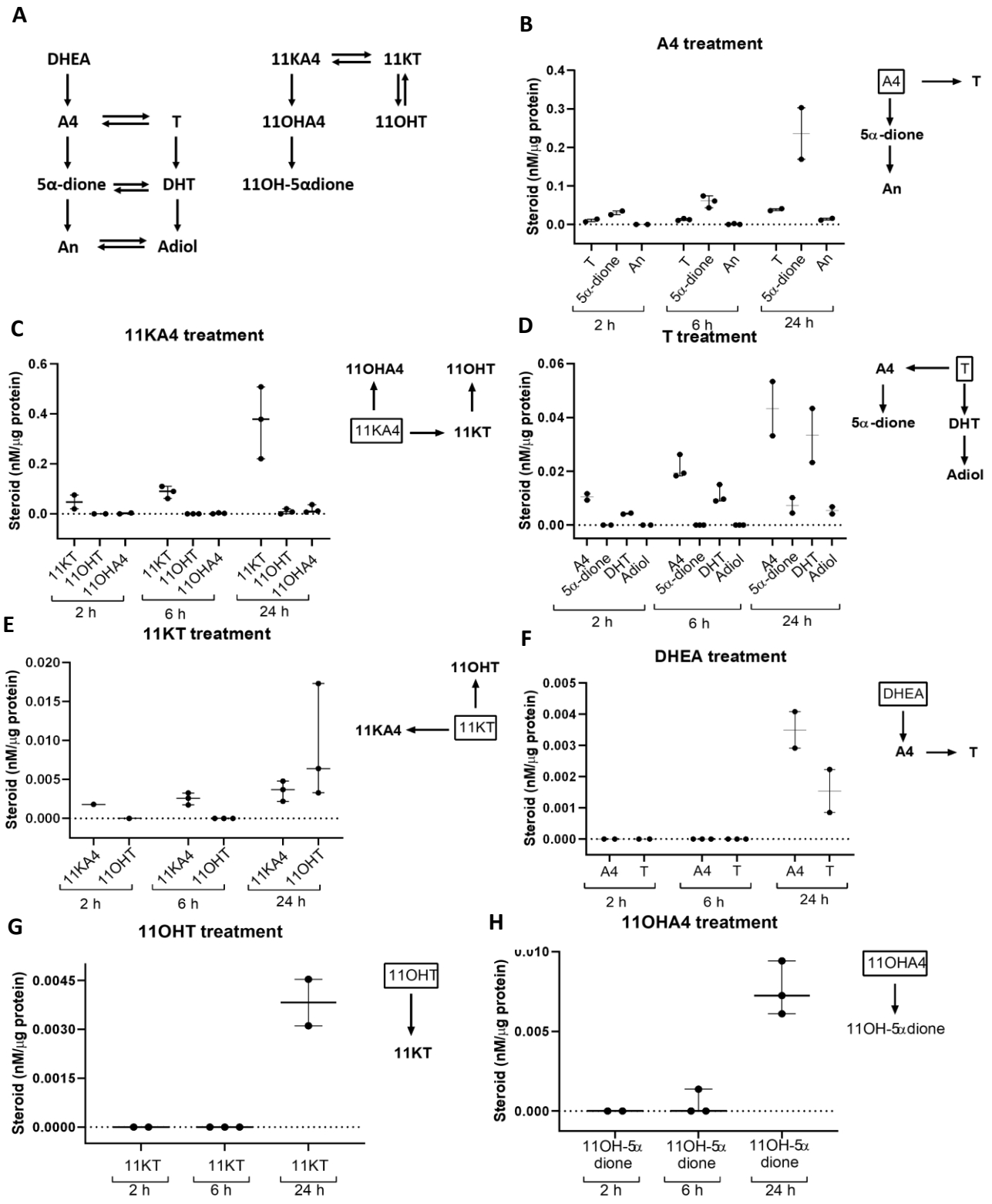

Figure 3.2 ***Ex vivo*** **time-dependent conversions of classic and 11-oxygenated androgens by PBMCs.** PBMCs were isolated from three healthy males (age range 22-28 years; BMI range 20.2-29.1 kg/m^2) and incubated with 100 nM of classic or 11-oxygenated androgens **(B-H)** for 2, 6 or 24 hours. The respective substrate is shown in a black box for each graph. Product formations was quantified by LC-MS/MS and normalised to the total protein content of the incubation. Individual data points for each donor, range and median are shown. Product concentrations below the limit of quantification are shown as 0.

3.3 No significant age-related differences were observed in androgen activation in PBMCs

To characterise the effect of age on androgen metabolism in PBMCs, *ex vivo* steroid conversion assays and gene expression analysis by qPCR were performed with PBMCs isolated from young men aged between 22-30 years (n=4-5; **Table 2.2**) and age-advanced men between 53-72 years (n=4-7; **Table 2.2**). No significant differences between the two age groups were observed for *ex vivo* androgen metabolism and the expression of genes encoding androgen-metabolising enzymes (**Figure 3.3**).

A4 and T were efficiently converted to their 5α-reduced products 5α-dione and DHT, respectively. Additionally, 5α-dione and DHT were further metabolised to An and Adiol, respectively, however at negligible levels (**Figure 3.3A and 3.3B**). The highest product formation observed from all substrates tested was the generation of 11KT from its precursor 11KA4 (**Figure 3.3C**). Thereby, a higher median 11KT generation in males aged over 50 years compared to males aged 18-30 years was observed indicating a trend for an increased androgen activation by AKR1C3 with age, however this was not significant. When PBMCs were incubated with 11KT (**Figure 3.3D**), only a minor generation of 11KA4 was produced (quantifiable in 8/12 incubations) compared to the generation of A4 from T (**Figure 3.3B**). HSD11B1 activity in PBMCs was indicated by the conversion of 11KA4 to 11OHA4 (quantifiable in 8/12 incubations) **(Figure 3.3C)** and the conversion of 11KT to 11OHT (quantifiable in 4/12 incubations) **(Figure 3.3D).** Compared to the observed 17β-hydroxysteroid dehydrogenase and 5α-reductase activities, HSD11B1 activity was negligible. Incubation with 11OHA4 resulted in the formation of 11-OH5αdione, due to 5α-reductase activity (**Figure 3.3E**) and incubation with 11OHT produced 11KT due to HSD11B2 activity (**Figure 3.3**). However, the efficiency of 11OHA4 and 11OHT conversions was minor in comparison to the other substrates tested.

Taken together, these results indicate that the reductive 17β-hydroxysteroid dehydrogenase activity of AKR1C3, which activates both classic (A4 to T) and 11-oxygenated androgens (11KA4 to 11KT), is the predominant reaction of androgen metabolism in male PBMCs. Additionally, the results demonstrate that 5α-reductase activity is only relevant for classic androgens, shown by the active

conversion of A4 to 5α-dione and T to DHT, and not for 11-oxygenated androgens with only negligible 5α-reduction of 11OHA4 to 11OH-5α-dione.

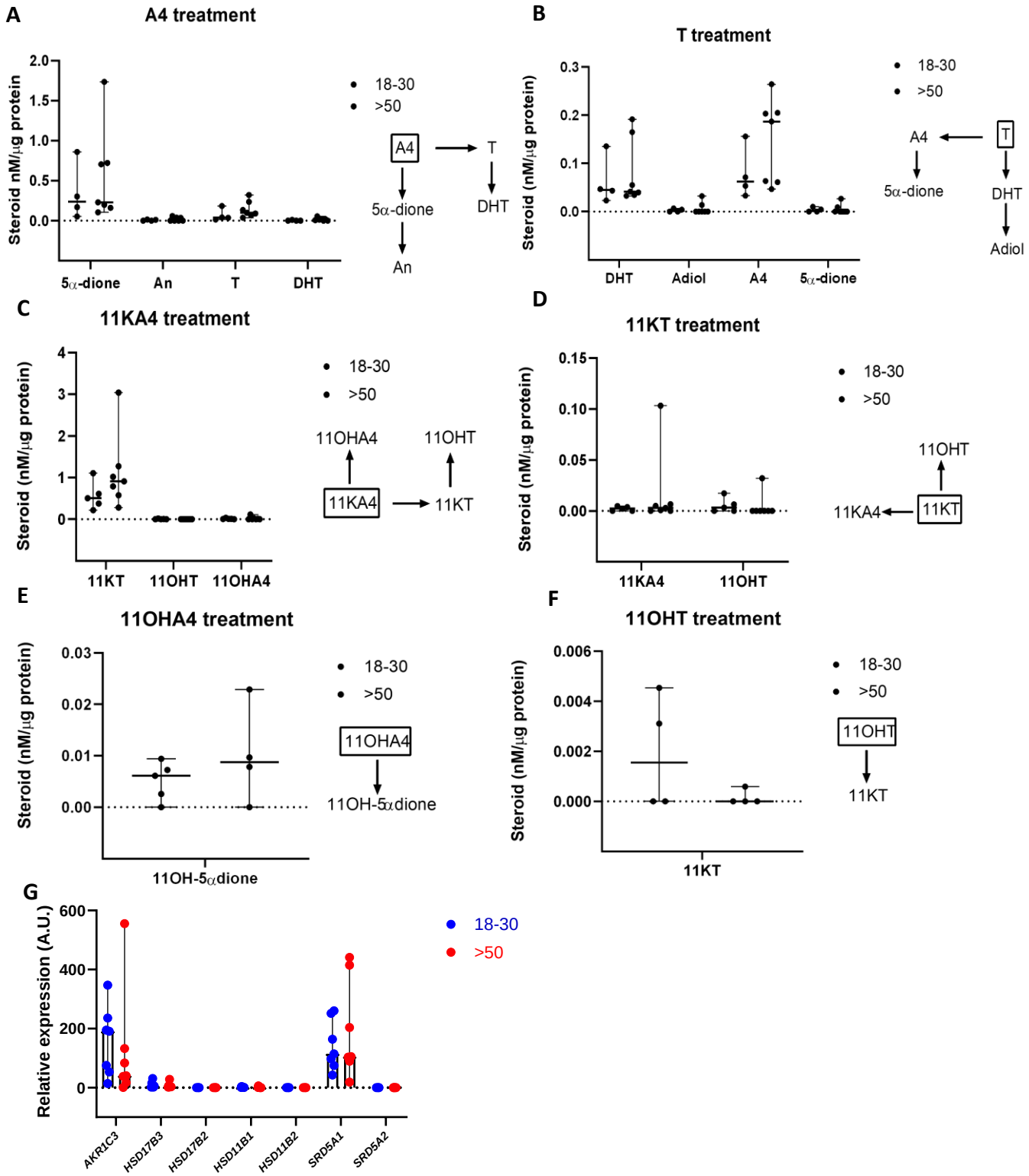

Figure 3.3 *Ex vivo* androgen metabolism in PBMCs from men aged 18-30 years and over 50 years. *Ex vivo* steroid conversion assays **(A-F)** were carried out in PBMCs isolated from healthy males aged 18-30 years (blue, n=4-5; age 22-30 years; BMI 20.2-29.1 kg/m^2) and over 50 years (red, n=4-7; age 53-72 years; BMI 21.2-30.4). The respective substrate is shown in a black box for each graph. Product formation was quantified by LC-MS/MS and normalised to the total protein content of the incubation. The individual data points, range and median are shown. Product concentrations below the limit of quantification are shown as 0. **(G)** The gene expression of enzymes relevant in PBMC androgen metabolism from healthy males aged 18-30 years (blue) and over 50 years (red) was assessed by qPCR, normalised to housekeeping genes HPRT1 and GAPDH. Arbitrary units (A.U.) were calculated as 1000*2^-ΔCt.

3.4 11-oxygenated androgens are the predominant androgens activated in PBMCs

After establishing the contribution of 17β-hydroxysteroid dehydrogenase activities to androgen-metabolism in PBMCs, we wanted to examine whether there was a preference for classic or 11-oxygenated androgen activation. As there were no significant differences between androgen metabolism in PBMCs from the two different age groups (**Figure 3.3**), the data from the entire male cohort was combined (n=8-12; age 22-72; BMI 20.2-30.4 kg/m^2) to compare the activation and inactivation of classic and 11-oxygenated androgens.

Figure 3.4A shows that after 24 hours, approximately eight times more of the active 11-oxygenated androgen 11KT was generated than the classic androgen T from their respective precursors 11KA4 and A4, which was statistically significant (p=0.001). **Figures 3.4A** and **3.4B** illustrate the active interconversion which takes place between A4 and T, due to both reductive and oxidative 17β-hydroxysteroid dehydrogenase activities. Importantly, significantly more A4 was generated from T compared to the generation of 11KA4 from 11KT (11KA4 quantifiable in 8/12 incubations) (**Figure 3.4B**, p=0.001). Additionally, the product/substrate ratios reflecting the steady state of activation and inactivation by 17β-hydroxysteroid dehydrogenase activities confirm the preference of the activation of 11-oxygenated androgens (**Figure 3.5A**). In conclusion, the greater formation of 11KT from 11KA4 than T from A4 in combination with the negligible back-conversion of 11KT to 11KA4 indicates that PBMCs preferentially activate 11-oxygenated over classic androgens. The product/substrate ratios for 5α-reductase activity in PBMCs reflect the substrate preference of SRD5A1 with a higher activity for the 5α-reduction of A4 (5α-dione/A4) than for T (DHT/T) (**Figure 3.5B**). Additionally, it reveals negligible 5α-reductase activity for 11OHA4 (11OH5α-dione/11OHA4). No 5α-reduction was observed for the 11-ketoandrogens, 11KA4 and 11KT.

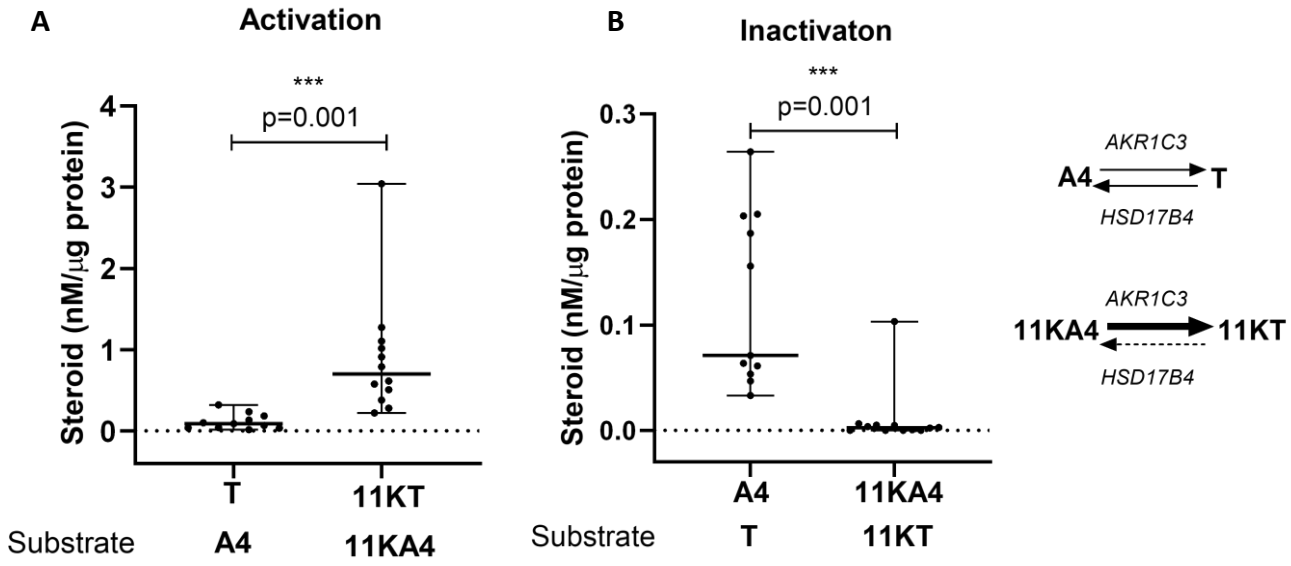

Figure 3.4 *Ex vivo* interconversions of the classic androgens A4 and T and the 11-oxygenated androgens 11KA4 and 11KT by PBMCs. PBMCs were isolated from healthy men (n=8-12; age range 22-72 years; BMI range 20.2-30.4 kg/m^2). PBMCs were incubated with 100 nM of substrates for 24 hours. **(A)** Activation of A4 and 11KA4 by reductive, activating 17β-hydroxysteroid dehydrogenases. **(B)** Inactivation of T and 11KT by oxidative, inactivating 17β-hydroxysteroid dehydrogenases. Product formation was quantified by LC-MS/MS and normalised to the total protein content of the incubation. The individual data points for each donor, range and median are shown. Product concentrations below the limit of quantification are shown as 0. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test. ** = P ≤ 0.05, ** = P ≤ 0.01, *** = p ≤ 0.001.*

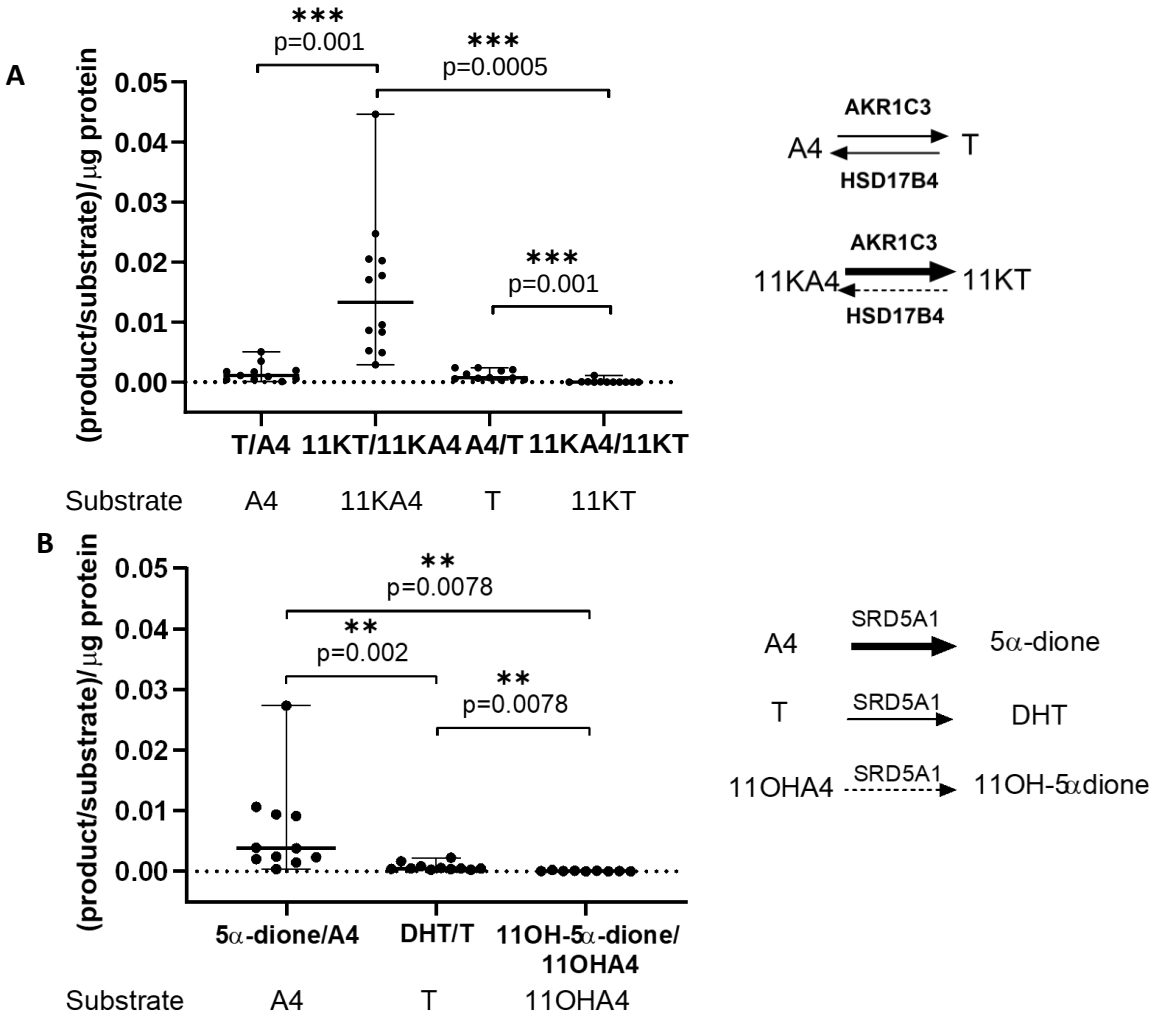

Figure 3.5 Product/substrate ratios reflecting the contributions of AKR1C3, HSD17B4 and SRD5A1 to classic and 11-oxygenated androgen metabolism in PBMCs.

PBMCs were isolated from healthy males (n=8-12; age range 22-72 years; BMI range 20.2-30.4 kg/m^2) and incubated with 100 nM A4, 11KA4, T, 11KT and 11OHA4 for 24 hours. Product/substrate ratios were calculated representing the enzymatic activity of **(A)** activating 17β-hydroxysteroid dehydrogenase activity by AKR1C3 and inactivating 17β-hydroxysteroid dehydrogenase activity by HSD17B4, **(B)** 5α-reductase activity by SRD5A1. Products and substrates were quantified by LC-MS/MS and their ratios were normalised to the total protein content of the incubation. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test. *ns = P > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, *** = p ≤ 0.001.*

3.5 No effect of insulin observed on androgen metabolism in PBMCs from female donors

In order to assess whether insulin regulates AKR1C3 activity, as established for adipose tissue (O'Reilly et al., 2017), PBMCs were prepared from blood of four healthy female volunteers (age range 21-32; BMI 20.1-22.8kg/m^2). Based on established protocols for the induction of AKR1C3 expression in adipocytes (O'Reilly et al., 2017), PBMCs were pre-treated with 10 nM insulin or vehicle for four hours prior to the steroid conversion assays in the presence or absence of insulin. When incubated with the classic androgen precursor A4, the conversion to T was similar in both insulin-treated and non-treated PBMCs (**Figure 3.6A**) and as were product/substrate ratios (T/A4) **(Figure 3.6B).** Similarly, when treated with the 11-oxygenated androgen precursor 11KA4, no significant changes in the generation of the active 11-oxygenated androgen 11KT (**Figure 3.6A**) and the product/substrate ratio (11KT/11KA4) **(Figure 3.6B)** were observed. Although no meaningful AKR1C3 activity above baseline was observed in this assay, there was little evidence that AKR1C3 activity was detected in either the control or insulin group, meaning it cannot be concluded that insulin does not affect AKR1C3 activity. qPCR analysis of *AKR1C3* expression after insulin treatment could not be performed due to the interruption of the project by the COVID-19 related closure of the laboratories.

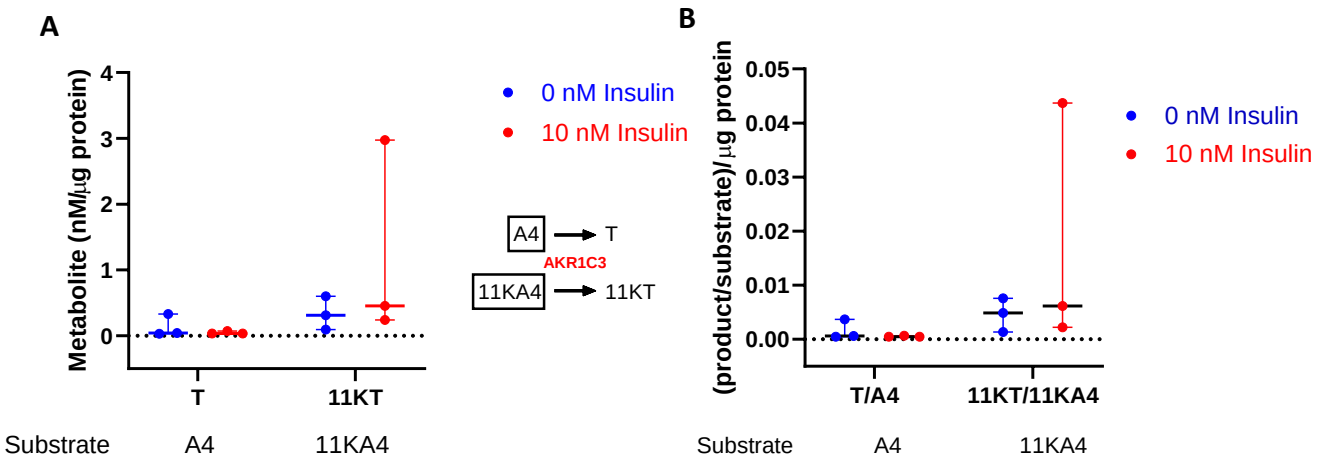

Figure 3.6 The effect of insulin on androgen precursor activation by AKR1C3 in PBMCs.

PBMCs from four healthy females (age range 21-32; BMI 20.1-22.8kg/m2) were pre-treated with 10 nM Insulin or vehicle and incubated for 4 hours followed by steroid conversion assays in the presence or absence of insulin. **(A)**. Product formation was quantified by LC-MS/MS and normalised to the total protein content of the incubation. The respective substrate is shown in a black box. The individual data points, range and median are shown. Product concentrations below the limit of quantification are shown as 0. **(B)** Steady state ratios of AKR1C3 products and substrates for classic (T/A4) and 11-oxygenated androgens (11KT/11KA4).

3.6 Natural killer cells are the major PBMC population of AKR1C3 expression and activity

After establishing AKR1C3 as the key androgen activating enzyme in PBMCs, responsible for T and 11KT production from A4 and 11KA4, respectively, it was consequently investigated whether a specific PBMC subpopulation was responsible for the observed AKR1C3 activity. To investigate the expression of *AKR1C3* in different PBMC populations, RNAseq gene expression data available at dice-database.org from 15 FACS-sorted PBMC subpopulations including B-cells, T-cell populations and NK cells (Schmiedel et al., 2018; dice-database.org) was analysed. As shown in **Figure 3.7A**, the expression of *AKR1C3* in NK cells was significantly higher ($p<0.0001$) than all other immune cell types investigated. To examine whether the NK cell population was also responsible for the AKR1C3 activity observed in the PBMC ex vivo incubations, PBMCs were isolated from an anonymous donor's leukocyte cone and NK cells were enriched from the isolated PBMCs. Steroid conversion assays with the AKR1C3 substrates A4 and 11KA4 were then carried out using the enriched NK cell preparation and the matched crude PBMC isolate containing NK cells and all subpopulations. For both A4 and 11KA4

incubations, AKR1C3 activity was higher in the incubation with enriched NK cells (n=4 technical replicates) compared to the matched crude PBMC isolate (n=1) (**Figure 3.7B**). **Figure 3.7B** also illustrates that like in PBMC incubations (**Figure 3.7A**), AKR1C3 activity in enriched NK cells also favours the production of the 11-oxygenated 11KT, which was 7.3 times higher than the production of classic T. Quantitative PCR was used to assess the relative *AKR1C3* expression in the crude PBMC isolate (containing all subpopulations including NK cells), the enriched NK cell preparation and the remaining mixed PBMC population after NK cell enrichment from the same donor. Both the crude PBMC isolate (containing NK cells and all other cell populations) and enriched NK cells have comparable levels of *AKR1C3 expression*, while the NK cell depleted PBMC mix has only minor *AKR1C3* expression (**Figure 3.7D**). Therefore, this suggests that the reductive, activating 17β-hydroxysteroid dehydrogenase activity of AKR1C3 observed in the PBMC *ex vivo* incubations is predominantly located in the NK cell subpopulation. As the comparison of NK cell AKR1C3 activity was relative to n=1 crude PBMC samples, this experiment would need to be repeated to ensure observations in this comparison are meaningful.

SRD5A1, the other enzyme besides AKR1C3 making a major contribution to classic androgen metabolism in PBMCs, showed the highest expression in monocytes followed by NK cells (**Figure 3.7D**). In keeping with this, incubations with the SRD5A1 substrates A4 and T showed similar SRD5A1 activity in the NK cells and the crude PBMC isolate (**Figure 3.7E**) and *SRD5A1* expression was similar in the crude PBMC isolate, the enriched NK cells and the remaining cells after NK cell isolation (**Figure 3.7F**).

Overall, these data show that PBMC subpopulations show differential expression of steroid-metabolising enzymes; NK cells have significantly higher *AKR1C3* expression compared to all other subpopulations whilst monocytes have the highest *SRD5A1* expression.

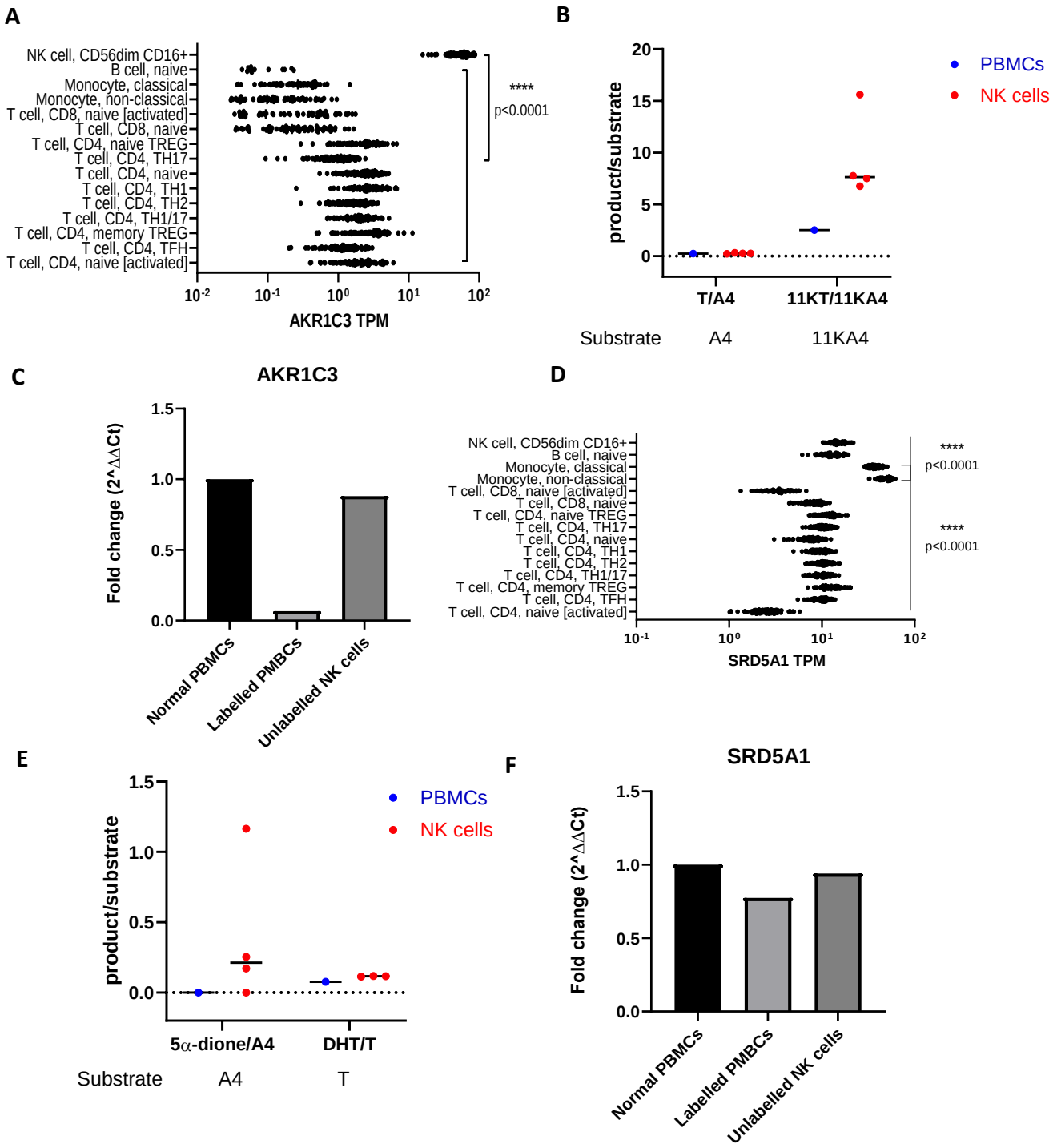

Figure 3.7 The expression and activity of AKR1C3 and SRD5A1 in natural killer cells.

RNAseq analysis of FACS-sorted PBMC subpopulations shows that **(A)** NK cells are the PBMC subpopulation with the highest *AKR1C3* expression and **(D)** monocytes are the PBMC subpopulation with the highest *SRD5A1* expression (n=91; data from dice-database.org). The Transcript Per Million (TPM) values are visualized for 15 blood cell types. Statistical analysis of AKR1C3 and SRD5A1 expression in the PBMC subpopulations was performed by one-way ANOVA followed by Tukey's multiple comparison test. **(B)** Incubations with A4 and 11KA4 showed higher AKR1C3 activity in NK cells (n=4 technical replicates) compared to mixed PBMC cells from the same donor (n=1) whilst incubations with A4 and T in NK cells (n=4 technical replicates) showed similar SRD5A1 activity in the NK cells and the crude PBMC isolate (n=1) **(E).** NK cells isolated using MACS negative selection (Miltenyi Biotec) were incubated with 100 nM substrate and product formation after 24 h was analysed by LC-MS/MS. The relative changes in gene expression for *AKR1C3* **(C)** and *SRD5A1* **(F)** was assessed by qPCR and normalised to the housekeeping genes HPRT1 and GAPDH. Fold changes were calculated as 2^ΔΔCt. *ns =P > 0.05, * = P =≤ 0.05, ** = P ≤ 0.01, *** = p ≤ 0.001, **** = P ≤ 0.0001.*

3.7 Lag time prior to the separation of cellular components from whole blood influences the androgen concentration measured in serum samples after separation

To assess whether the amount of time that whole blood is left unseparated influences the measurement of serum steroid concentrations, whole blood samples were collected from three male and three female volunteers **(Table 2.2).** Samples were left unseparated at room temperature for up to 24 hours and after the specified incubation time, the blood samples were centrifuged for the separation of serum from the cellular components. The androgen levels in the serum were quantified by LC-MS/MS. An increase in the concentration of the AKR1C3 product 11KT was observed over 24 hours (**Figure 3.8A**). After two hours, the increase in 11KT started to be statistically significant (p=0.01-0.05), and after 24 hours a median relative increase of 44.3% was seen. In line with this, a decrease in the concentrations of 11KA4, the substrate for 11KT generation by AKR1C3, was seen, with a median relative decrease of 33.9% after 24 hours (**Figure 3.8B**). The decrease in 11KA4 did not reach statistical significance. As shown in **Figure 3.8C** decreases in the concentration of A4, another AKR1C3 substrate, were observed, with the time points 1, 4, 6 and 8 hours being statistically significant (p=0.02-0.03). After 24 hours, the median relative decrease in the concentration of A4 was 19.2%. No changes over time were observed for T, the product of A4 conversion by AKR1C3 (**Figure 3.8D**). Amongst the other androgens measured (DHEA, DHT, An and 11OHA4; **Figures 3.8E-G**), only 11OHA4 displayed significant changes over time (p=0.03-0.04), with the highest median relative decrease of 41.3% observed after 8 hours **(Figure 3.8H)**. Overall, the changes in the concentration observed when whole blood is left unseparated suggests that there is relevant androgen metabolism occurring in whole blood. The decrease of the AKR1C3 substrate 11KA4 in combination with the significant increase of the respective product 11KT (**Figure 3.8B and 3.8A**) indicates relevant AKR1C3 activity in blood cells.

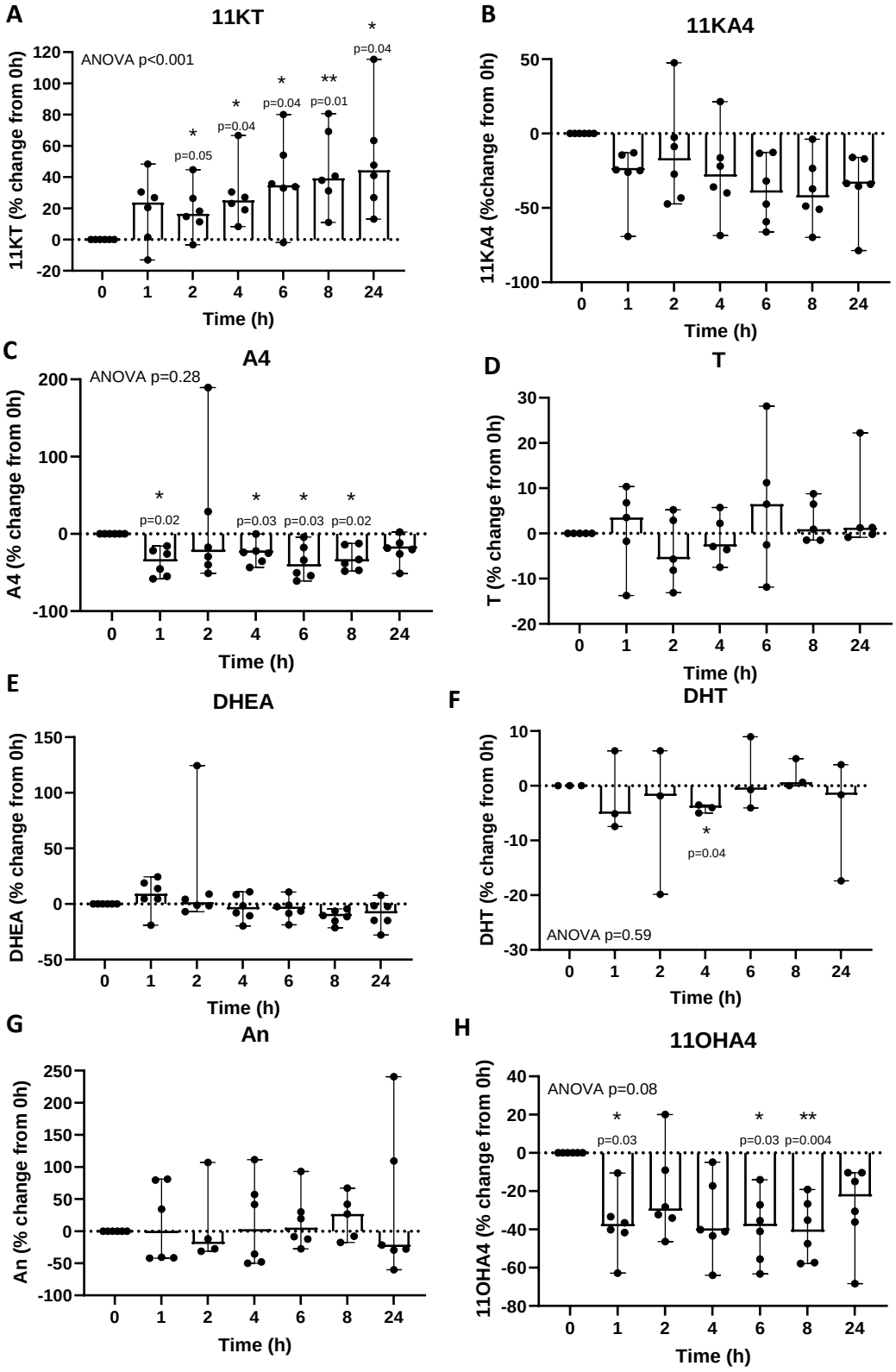

Figure 3.8 Percentage changes in serum steroid concentrations after incubation of the whole blood compared to their baseline concentration (0 h). 0 h samples were separated by centrifugation within 1h after collection. The other samples were incubated as whole blood at room temperature for either 1, 2, 4, 6, 8 or 24 hours until separation. **(A)** n=6 for 11KT **(B)** n=6 for 11KA4 **(C)** n=6 for A4 **(D)** n=5 for T **(E)** n=6 for DHEA **(F)** n=3 for DHT **(G)** n=6 for An **(H)** n=6 for 11OHA4. The range and median of the individual data points are shown. Statistical analysis was performed on the absolute concentrations (nM) using ANOVA followed by Dunnett's Multiple Comparison test to compare each time point against 0 hours.

4. Discussion

PBMCs preferentially activate 11-oxygenated androgens over classic androgens via AKR1C3

It has been shown that androgen signalling has immunosuppressive properties, and can down-regulate antibody production, T cell proliferation, as well as stimulate anti-inflammatory cytokines (Trigunaite et al., 2015; Ben-Batalla et al., 2020). Within peripheral tissues, local androgen activation from adrenal androgen precursors makes a major contribution to human androgen receptor (AR) activation (Schiffer et al., 2019). The purpose of this study was to examine the metabolism of 11-oxygenated and classic androgens in PBMCs, to assess whether AKR1C3 favours the activation of 11-oxygenated androgens over classic androgens and to investigate whether 11KT is the predominant androgen synthesised in PBCMs. Hammer et al (2005) has shown that human PBMCs activate the classic androgen precursor A4 to T via AKR1C3 activity and that T can be further activated to the most potent human androgen DHT. Additionally, they also confirmed that AKR1C3 is the reductive 17β-hydroxysteroid dehydrogenase responsible for androgen activation in PBMCs.

For the first time this project has shown that in males, PBMCs favour the activation of the 11-oxygenated androgen precursor 11KA4 to its active counterpart 11KT over the activation of the classic androgen precursor A4 to T. Pretorius et al (2016) showed that 11KT binds to and activates the human AR with an affinity, potency and efficacy comparable to T, establishing 11KT as an active androgen. It has also been shown *in vitro* using cell lines recombinantly overexpressing AKR1C3, that AKR1C3 has a significantly higher catalytic efficiency for the generation of 11KT than T (Barnard et al., 2018). This project confirmed this preference of AKR1C3 to activate 11-oxygenated androgens for the first time in human male cells *ex vivo.* Results from this study showed that PBMCs generate approximately 8-fold more of the 11-oxygenated androgen 11KT than the classic androgen T from their respective precursors 11KA4 and T, making 11KT the quantitively most important active androgen generated within male PBMCs. These results suggest that within males, 11KT may be the more physiologically relevant androgen than T in peripheral cells and tissues expressing AKR1C3. This project confirmed

that HSD17B4 is the primary oxidative, androgen-inactivating 17β-hydroxysteroid dehydrogenase in PBMCs (Hammer et al., 2005), while expression of HSD17B2, the other major androgen-inactivating 17β-hydroxysteroid dehydrogenase was not observed. This study found that, when PBMCs are incubated with the active androgens T and 11KT, the back-conversion to their respective inactive precursors A4 and T is relevant only for T, whilst the inactivation of 11KT to 11KA4 is negligible. The increased activation of 11-oxygenated androgens compared to classic androgens by AKR1C3, along with the minor inactivation of 11-oxygenated androgens compared to classic androgens by HSD17B4 indicates that male PBMCs preferentially activate 11-oxygenated androgens. These findings are consistent with the study by Barnard et al (2018), which showed that *in vitro* HSD17B2 has similar catalytic efficiencies for 11KT and T conversion, but that increased ratios of AKR1C3 to HSD17B2 favour the activation of 11-oxygenated androgens due to the preferred activation of 11KA4 to 11KT by AKR1C3. This work contributes to the growing body of evidence on the key role of AKR1C3 for the regulation of androgen signalling in different peripheral tissues. Other tissues in which androgen regulation by AKR1C3 has been shown to relevant include adipose tissue (O'Reilly et al., 2017; Quinkler et al., 2004), endometrium (Gibson et al., 2016) and prostate (Barnard et al., 2018). To follow on from the findings in this study, next steps would also be to examine whether 11-oxygenated androgens are also preferentially activated *in vivo.* For example, oral doses of a classic androgen precursor such as DHEA and an 11-oxygenated androgen precursor such as 11OHA4 could be given to participants and urine metabolites could be quantified.

For this study, it was also planned to investigate AKR1C3 activity in PBMCs from healthy women and women with PCOS. However, due to the COVID-19 pandemic and closure of the research facilities, these experiments could not be performed meaning the data generated is only representative of male PBMCs and cannot be generalised to both sexes. It is vital to follow on from this study by examining AKR1C3 activity in female PBMCs to ensure potential differences between male and female AKR1C3 activity is recorded.

5α-reduction by SRD5A1 does not contribute to 11-oxygenated androgen metabolism in PBMCs

This study has reinforced previous findings by Hammer et al (2005) that 5α-reduction contributes to the activation of classic androgens in PBMCs by generating DHT. It has previously been shown that SRD5A1 is the major 5α-reductase in PBMCs (Hammer et al., 2005), and this project confirms the expression of SRD5A1 in PBMCs, while SRD5A2 was undetectable. The substrate preference of 5α-reduction observed in male PBMCs with higher activity for the conversion of A4 to 5α-dione than for T to DHT is consistent with the previously established substrate preference of SRD5A1 (Thigpen et al., 1993). Pretorius et al (2016) showed *in vitro* using promoter reporter assays that 11KDHT can activate the AR with potency and efficacy comparable to DHT. However, it has been uncertain to date whether the generation of 11KDHT by 5α-reduction of 11KT is relevant in physiological conditions. In this study, no relevant 5α-reduction of 11-oxyganted androgens was observed, with only negligible 5α-reduction of 11OHA4 and no 5α-reduction of 11KA4 to 11K-5α-dione or 11KT to 11KDHT. Barnard et al (2020) have shown *in vitro* with cell lines overexpressing steroid reductases, that 11KT is efficiently 5α-reduced to 11KDHT by SRD5A2 only, but not by SRD5A1. This project now confirms this with human male cells *ex vivo* by demonstrating that 11KT is not 5α-reduced by PBMCs, which do not have SRD5A2, but levels of SRD5A1 which efficiently convert T to DHT. This establishes that 11KDHT is not a relevant metabolite of 11KT in PBMCs. Hence, while 5α-reduction can further potentiate the AR activation by classic androgens in PBMCs, the activity of 11-oxygenated androgens is predominantly regulated by AKR1C3.

Donor age did not significantly affect androgen activation in male PBMCs in this study

When comparing androgen metabolism in PBMCs isolated from men aged 18-30 years and above 50 years with similar BMIs, no significant influence of age on androgen activation was observed. These results contrast with a published study which described an increase in AKR1C3 and SRD5A1 activity in males aged over the age of 50 compared to males aged 18-30 (Hammer et al., 2005). The discrepancies in results can likely be attributed to the small cohort size of both studies, as well as the different assays

used to quantify the steroids (LC-MS/MS in this study *vs* thin layer chromatography in Hammer et al). Although no significant differences in age were found, our results displayed a higher median for the activation of 11KT from 11KA4 in men aged over 50 compared to the younger age group possibly indicating a trend for an increased AKR1C3 activity. Circulating levels of classic androgens have been shown to decline with age, whilst levels of 11-oxygenated androgens remain comparatively constant throughout an adult's lifespan (Nanba et al., 2019; Davio et al., 2020). Therefore, the peripheral activation of 11-oxygenated androgens is favoured over the activation of classic androgens not only by the substrate preference of AKR1C3 for 11-oxygenated androgens, but also stable availability of 11-oxygenated androgen precursors in circulation throughout adulthood. It is important to properly establish the effect of age on androgen metabolism, this requires a larger cohort size in the male groups, but this could also be examined in pre-menopausal and post-menopausal women.

Effect of ex vivo insulin treatment on androgen metabolism in PBMCs not observed

Polycystic ovary syndrome is associated with androgen excess, and it has been shown that women with PCOS have significantly increased concentrations of serum T and 11KT compared to controls (O'Reilly et al., 2017). Moreover, insulin resistance and hyperinsulinemia are also observed in women with polycystic ovary syndrome (Rojas et al., 2014). It has been shown that AKR1C3 expression and activity in adipose tissue are upregulated by insulin, establishing a direct link between hyperinsulinemia and androgen excess (O'Reilly et al., 2017). Hence, this study aimed to examine whether insulin also increases AKR1C3 activity in PBMCs. PBMCs were treated with insulin prior to and during *ex vivo* androgen conversion assays following protocols that induced a transcriptional response and an increase in *AKR1C3* expression and activity in a human preadipocyte cell line (O'Reilly et al., 2017). This study found no significant changes in AKR1C3 activity when compared to vehicle treated PBMCs. However, due to the COVID-19 pandemic and related closure of the university laboratories, the results presented on the effect of insulin on AKR1C3 activity in PBMCs have limitations that need to be considered. PBMCs from only a limited number of donors (n=3) could be studied. Although no

meaningful AKR1C3 activity was observed in this assay, there was little evidence that AKR1C3 activity was detected in either the control or insulin group, meaning it cannot be concluded that insulin does not affect AKR1C3 activity. Additionally, it could not be confirmed within this project whether the concentration and duration of the insulin treatment successfully caused activation of insulin signalling in PBMCs and changes in *AKR1C3* gene expression. To confirm that the insulin treatment resulted in the activation of intra-cellular signalling, the phosphorylation status of intra-cellular signalling proteins involved in insulin signalling, such as Akt or IRS1 would have to be examined by Western Blot (Boucher et al., 2014) and gene expression of known insulin responsive genes and *AKR1C3* by qPCR.

Androgen activation in PBMCs from women with polycystic ovary syndrome

AKR1C3 is upregulated in adipose tissue of women with PCOS contributing to the generation of local androgen excess (O'Reilly et al., 2017; Quinkler 2004). For this study, it was planned to investigate if the upregulation of AKR1C3 in PCOS is a systemic phenomenon also taking place in tissues other than adipose and hence, if increased androgen activation via AKR1C3 is also observed in PBMCs from women with PCOS compared to healthy women. As previous literature has shown, 11KT, the product of 11-oxygenated androgen activation by AKR1C3, is the dominant circulating active androgen in PCOS (O'Reilly et al., 2017). Therefore, the collection of blood for PBMC isolation had been built into the protocol of an *in vivo* study lead by the supervisor of this thesis, Professor Wiebke Arlt, performing an in-depth metabolic phenotyping of women with PCOS and age- and BMI-matched controls. This would have allowed for a detailed comparison of androgen activation *ex vivo* and expression of AKR1C3 in PBMCs. However, due to the COVID-19 pandemic and closure of the research facilities, the experiments could not be performed as the *in vivo* study was postponed, and this MSc project had to finish five months earlier than expected. Female androgen excess is often associated with obesity (Pasquali et al., 2019). To understand if peripheral tissues, other than adipose, contribute to the hyperandrogenism observed in obesity, it had additionally been planned to compare androgen activation in PBMCs isolated from lean women (BMI <25 kg/cm^2) and overweight women (BMI >30

kg/cm^2). Lean women had already been recruited for the experiments assessing the effect of insulin and the control cohort from the PCOS *in vivo* study would have served as obese comparison.

Natural killer cells are the major PBMC subpopulation of AKR1C3 activity

NK cells are vital for eliminating transformed or viral-infected cells and elicit these effects by secreting cytokines and chemokines and inducing cell cytotoxicity (Hazeldine et al., 2013). It has been shown that androgens can regulate NK cells by supressing NK cell proliferation and decreasing NK cell cytotoxicity (Page et al., 2006; Ben-Batalla et al., 2020). The results from this project show that among the PBMC subpopulations, NK cells show significantly higher expression and activity of AKR1C3. Primary adrenal insufficiency (PAI) is associated with an increased risk for both fatal and non-fatal infections, along with significantly impaired NK cell cytotoxicity (Bancos et al., 2017). Independent of whether a PAI patient is receiving regular DHEA replacement therapy, NK cell cytotoxicity is generally reduced excluding DHEA deficiency as the cause of the decreased NK cell cytotoxicity (Bancos et al., 2017). Our results show that 11-oxygenated androgen precursors, which are of adrenal origin, are more relevant than classic androgen precursors for the generation of active androgens in PBMCs, and predominantly in NK cells. Therefore, this suggests that a deficiency of adrenal 11-oxygenated androgen precursors could be responsible for the associated reduced NK cell cytotoxicity in PAI. However, additional work is required to characterise the effects of 11-oxygenated androgens and their activation by AKR1C3 on NK cell function including whether 11-oxygenated androgens can rescue the impaired cytotoxicity observed in primary adrenal insufficiency. Due to COVID-19 and the premature ending of this project, the data presented for AKR1C3 activity in NK cell has a number of limitations. The experiments were performed with cells from one donor only. The results of the *ex vivo* steroid conversion assays could not be normalised to the protein content of the incubations, because the protein quantification could not be performed. However, all incubations contained equal numbers of cells and no significant variations of the protein content are expected. Additionally, the NK cell preparation used to determine AKR1C3 activity in comparison to the matched crude PBMC isolate

could not be assessed for purity. Protocols for flow cytometry experiments with the NK cell specific surface markers CD56 and CD16 had been developed but could not be applied to the NK cell preparation used to study androgen metabolism. However, the NK isolation was performed with a validated, commercial MACS® negative selection kit following the manufacturer's instructions and activity results are consistent with the RNAseq data from dice-database.org, which show significantly higher *AKR1C3* expression in NK cells compared to all other PBMC subpopulations, which gave reassurance that cell isolation was performed accurately.

Analysis of RNAseq data from dice-database.org (Schmiedel et al., 2017) showed that *SRD5A1* has highest expression in monocytes compared to the other PBMC subpopulations, whilst *AKR1C3* has highest expression in NK cells. This project showed that in PBMCs, the 5α-reductase activity of SRD5A1 is only relevant for the activation of classic androgens, whilst AKR1C3 favours the activation of 11-oxygnenated androgens. This suggests that different PBMC subpopulations favour different androgen activating pathways depending on the differential expression of genes encoding androgen-metabolizing enzymes, with NK cells favouring the activation of classic androgens via SRD5A1 and NK cells favouring the activation of 11-oxygenated androgens by AKR1C3. However, to confirm this hypothesis, *ex vivo* steroid conversion assays with purified monocytes are required to establish that the differential gene expression compared to NK cells results in a different preference for the metabolism of androgens.

Lag time prior to the separation of cellular components leads to an increase in serum 11KT

The preference of PBMCs to generate 11KT from 11KA4 via AKR1C3 is illustrated in the stability of steroid levels in blood samples, when left unseparated. This project found that when whole blood is left unseparated from cellular components, there is a significant increase in 11KT serum levels after 2 hours, which is accompanied by a non-significant decrease in 11KA4 levels. This suggests that in a clinical setting, blood samples for the measurement of 11-oxygenated androgens should be processed within 2 hours of collection. 11KT is the dominant circulating active androgen in polycystic ovary

syndrome (O'Reilly et al., 2017) and congenital adrenal hyperplasia (Bacila et al., 2019; Turcu et al., 2016) and a biomarker of androgen excess. These results show that levels of 11KT are subject to change in whole blood samples if timely processing of the sample is not guaranteed and therefore salvia, which is cell-free, may be a more suitable sample for the measurement of 11KT in diagnosis (Bacila et al., 2019; Schiffer et al., 2019). As opposed to previous findings by Hawley et al (2020), which showed a significant increase in serum T over time, this study did not observe an increase in serum T in this study. Although both studies used LC-MS/MS to detect serum steroid levels, this inconsistency may be as a result of the small cohort sizes (14 participants in Hawley et al and 7 participants in this project) in combination with the low activity of AKR1C3 for the generation of T compared to 11KT.

Conclusion

This study has demonstrated that that within male peripheral blood mononuclear cells, AKR1C3 preferentially activates the 11-oxygenated androgen 11-ketotestosterone over the classic androgen testosterone. This study also showed that within the PBMC subpopulations, natural killer cells are the major source of AKR1C3 activity and expression **(Figure 4.1)**. Altogether, the results from this project imply that within male PBMCs, 11KT is the quantitively most important active androgen to be generated from its inactive precursor.

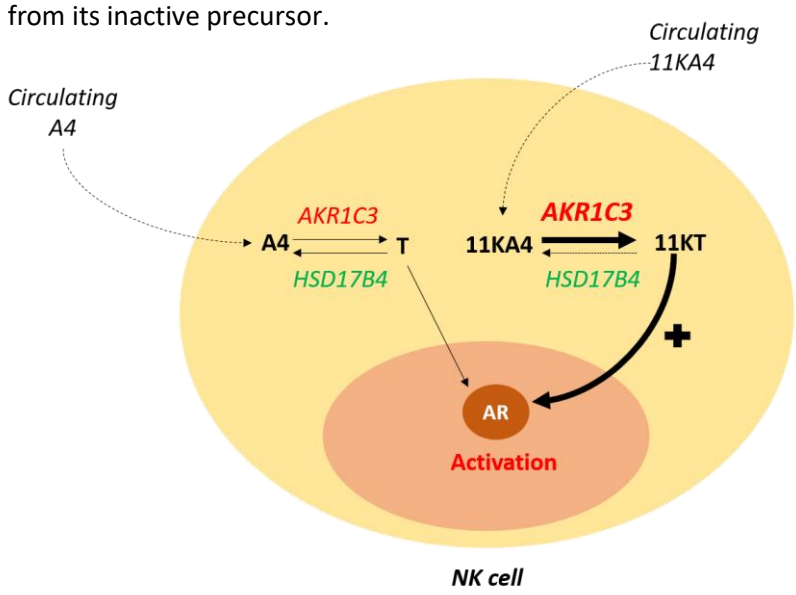

Figure 4.1 Local androgen activation by AKR1C3 in NK cells favours the production of the 11-oxygenated androgen 11KT over the classic androgen T. Circulating classic (A4) and 11-oxygenated androgen precursors (11KA4) are taken up by NK cells and metabolised by the activating (AKR1C3) and inactivating (HSD17B4) 17β-hydroxysteroid dehydrogenases. Due to the high activity of AKR1C3 for the generation of 11KT from 11KA4 and the resulting high intracellular concentrations of 11KT, 11KT is proposed to be the major mediator of AR signalling in NK cells. Original figure.

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