Dissertation

Androgen receptor and immune activation: analysis of the role of B cells as mediators of polycystic ovary syndrome

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Statutory Declaration

“I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice “.

Angelo Ascani

Tromsø, September 2024

Disclosures

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The role of B cells in immune cell activation in polycystic ovary syndrome.

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Abbreviations

17-OHPE	17-hydroxypregnenolone
17α-OH	17α-hydroxylase
17β-HSD	17β-hydroxysteroid dehydrogenase
A4	androstenedione
AAMs	alternatively activated macrophages
AE-PCOS	Androgen Excess and Polycystic Ovary Syndrome Society
AITD	Autoimmune thyroid disorders
AKR1C3	aldoketo reductase type 3
AMH	anti-Müllerian hormone
aNAV	activated naive cells
APCs	antigen-presenting cells
AR	androgen receptor
ARKO	Androgen Receptor knockout
ARTs	adipose resident T cells
ASRM	American Society for Reproductive Medicine
ATMs	adipose tissue macrophages
BAFF	B-cell activating factor
BAFFR	B-cell activating factor receptor
BAT	brown adipose tissue
BCR	B cell receptor
BMI	Body Mass Index
CAMs	classically activated macrophages

cDCs	conventional dendritic cells
CLSs	crownlike structures
cNK	circulating natural killer T cells
CRP	C-reactive protein
CSR	class-switch recombination
DCs	dendritic cells
DHEA	dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
DIO	diet-induced obese
DN	Double negative
DNA	Deoxyribonucleic acid
E1	estrone
E2	estradiol
ER	estrogen receptor
ESHRE	European Society of Human Reproduction and Embryology
FAI	Free Androgen Index
FF	follicular fluid
FFAs	free fatty acids
Fo	Follicular
FPG	fasting plasma glucose
FPI	fasting plasma insulin
FRCs	fibroblastic reticular cells

FSH	follicle-stimulating hormone
GCs	germinal centres
GnRH	gonadotropin releasing hormone
GPER	G protein-coupled estrogen receptor
GWAS	genome-wide association studies
HA	Hyperandrogenism
HDL	high-density lipoprotein
HFD	high fat diet
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HPG	hypothalamic–pituitary–gonadal axis
IGF-1	insulin-like growth factor 1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1Ra	IL-1 receptor antagonist
ILCs	Innate Lymphoid Cells
iNKT	invariant natural killer T cells
IR	Insulin resistance
IRS-1	insulin receptor substrate-1
iTregs	induced regulatory T cells
LC-MS	Liquid chromatography–mass spectrometry
LDL	low-density lipoproteins
LDLR	low-density lipoprotein receptor
LH	luteinizing hormone

LPS	lipopolysaccharide
LTi	lymphoid tissue-inducer
LTi	lymphoid tissue-inducer
MAPK	mitogen-activated protein kinase pathway
MCP-1	monocyte chemoattractant protein-1
mFG	modified Ferryman-Gallwey score
MIP	macrophage inflammatory protein
MS	multiple sclerosis
MZ	marginal zone
Nabs	natural antibodies
NCAH	non-classic adrenal hyperplasia
NCR	natural cytotoxicity receptor
NFκB	nuclear factor κB
NIH	National Institutes of Health
NMDA	N-methyl-D-aspartate
nTregs	naturally occurring regulatory T cells
oGTT	oral glucose tolerance test
oxLDL	oxidized low-density lipoprotein
PC	plasma cells
PCOM	polycystic ovarian morphology
pDCs	plasmacytoid dendritic cells
PE	pregnenolone
PI3K	phosphoinositide 3-kinase

PNA	Prenatally androgenized
RA	rheumatoid arthritis
RAG	Recombination activating gene
ROS	reactive oxygen species
SFAs	saturated fatty acids
SHBG	Sex hormone-binding globulin
SHM	somatic hypermutation
SLE	systemic lupus erythematosus
T2D	Type 2 Diabetes
TC	triglycerides
Th1	T-helper type 1
Th2	T-helper 2
TLR	Toll-like receptors
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cells
Trm	tissue-resident memory T cell
trNK	tissue-resident natural killer T cells
TSHR	receptor for thyrotropin
TSLP	thymic stromal lymphopoietin
uNK	uterine natural killer T cells
VLDL	very-low-density lipoproteins
WAT	white adipose tissue

Zusammenfassung

Das polyzystische Ovarsyndrom (PCOS) wurde mit Schwankungen der B-Zell-Frequenzen in Verbindung gebracht, obwohl der Mechanismus nach wie vor unklar ist. Diese lebenslange Entzündungskrankheit ist durch Störungen der Fortpflanzung und des Stoffwechsels gekennzeichnet, und frühere Versuche, eine Autoimmunursache zu ermitteln, waren relativ uninformativ. Das Ziel dieser Arbeit war zweierlei: festzustellen, ob selbstreaktive B-Zellen kausale Auswirkungen haben, und die allgemeinen Auswirkungen einer hohen Androgenexposition auf den Phänotyp der B-Zellen, insbesondere der doppelt negativen (DN) B-Gedächtniszellen, sowie auf die zirkulierenden Antikörpertiter zu untersuchen.

Hier zeigen wir, dass veränderte Frequenzen von B-Zellen eine direkte Auswirkung der Androgenrezeptor-Aktivierung sind und, was noch wichtiger ist, dass sie möglicherweise keine zentralen Mediatoren der PCOS-Pathologie sind. Tatsächlich weisen PCOS-hyperandrogene Frauen im Vergleich zu Kontrollpersonen eine erhöhte Häufigkeit von altersassoziierten DN CD27- IgD- B-Gedächtnissen auf, was auch mit erhöhten Spiegeln von zirkulierendem Immunglobulin M (IgM) einhergeht. Nach dem Transfer in weibliche Wildtyp-Mäuse führt humanes Serum-IgG vor allem zu einer Zunahme des Körpergewichts, während RAG1-Knock-out-Mäuse, denen reife B- und T-Zellen fehlen, keinen PCOS-ähnlichen Phänotyp entwickeln. B-Zell-Veränderungen im peripubertären Dihydrotestosteron (DHT)-induzierten PCOS-ähnlichen Mausmodell wurden durch die gleichzeitige Behandlung mit Flutamid, einem Androgenrezeptor-Antagonisten, verhindert. Schließlich entwickeln B-Zell-defiziente Mäuse, wenn sie DHT ausgesetzt sind, einen verschlimmerten PCOS-ähnlichen Phänotyp mit gestörter Glukose-Homöostase und einem höheren Anteil an Fettgewebe.

Insgesamt beweisen diese Ergebnisse, dass das Fehlen von B-Zellen nicht vor der Entwicklung eines PCOS-ähnlichen Phänotyps schützt, und deuten auf eine potenziell unerkannte doppelte Rolle der B-Zellen im spezifischen Umfeld der PCOS-assoziierten Entzündung hin, die sowohl schützend als auch entzündungsfördernd ist. Diese Ergebnisse drängen auf weitere Studien zur Aktivierung des angeborenen und

adaptiven Immunsystems bei PCOS, um entzündliche Anomalien besser behandeln zu können, die mit zahlreichen Begleiterkrankungen wie Diabetes und Immunthyreopathien einhergehen, die bei Frauen mit PCOS weit verbreitet sind.

Figure Legends

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Supplementary Fig. 1 Transfer of human IgG from women with PCOS to RAG 1$^{-/-}$ a. Estrous cycles in RAG1 -/- mice **b.** Estrous cycles in mice receiving Control IgG c. Estrous cycles in mice receiving PCOS IgG **d.** Weekly Body Weight recordings **e.** EcoMRI results for body fat and lean mass composition **f.** OgTT **g.** Fasting glucose. All bars indicate means; circles represent individual mice. Unpaired Student's t-test for analysis of anogenital distance difference between groups, as well as EchoMRI results and fasting glucose; two-way ANOVA with Sidak's post-hoc test for analysis of weekly BW recordings and blood glucose throughout the study. *P<0.05, **P<0.01, ***P<0.001.

Abstract

Polycystic ovary syndrome (PCOS) has been associated with variations in B cell frequencies although the mechanism remains unclear. This lifelong inflammatory disease is defined as a reproductive and metabolic disorder, and previous efforts to determine an autoimmune origin uncovered relatively ambiguous and incomplete results. The aim of this thesis was twofold: to establish, if self-reactive B cells have causal effects, and to investigate the overall impact of high androgen exposure on B cell phenotypes, particularly on age-associated Double Negative (DN) B memory cells. This atypical subset commonly expands with immunoscenescence, but studies suggest they also play an integral role in various diseases, generating autoimmunity or acting as immunosupressors. Here we show a direct link between altered frequencies of B cells and activation of the androgen receptor, and most importantly that they might not play a leading role in the development of PCOS pathology. Indeed, PCOS-hyperandrogenic women have higher frequency of atypical DN $CD27^-$ IgD^- B memory compared to controls, along with a distinct increase of serum immunoglobulin M (IgM). When transferred into female wild type mice, human-derived serum IgG induces mainly increased body weight, while recipient RAG1 knock-out mice lacking mature B and T cells do not to develop any PCOS-like phenotype. B cell variations in peripubertal dihydrotestosterone (DHT)-induced PCOS-like mouse model were prevented by concomitant treatment with an androgen receptor antagonist, flutamide. Lastly, when exposed to DHT, B cell-deficient mice present an aggravated PCOS-like phenotype with impaired glucose homeostasis and higher degree of adipose tissue.

Altogether, these results prove that there is no inherent protective effect to the development of a PCOS-like phenotype from the depletion of B cells and rather suggest a potentially unrecognized dual role for these cells in the specific setting of PCOS-associated inflammation, both protective and proinflammatory. These findings call for additional research on immune system activation in PCOS for better management of the associated comorbidities such as diabetes and immunothyropathies, commonly noted in women affected with PCOS.

1. INTRODUCTION

1.1 Polycystic Ovary Syndrome

Polycystic Ovary Syndrome (PCOS) is currently the most common endocrine and metabolic condition affecting 5-18% of women in reproductive age (1) inducing chronic inflammatory state with potential autoimmune complications (2). By definition, it is described by the combined presence of 3 diagnostic features: hyperandrogenism (HA), irregular menstrual cycles and polycystic ovaries (3,4). A concurrent adverse metabolic profile which can be further aggravated by obesity is a very common aspect of PCO syndrome, with 50-70% of women being insulin resistant (IR) (5,6). While addressing lifestyle may very often result in an adequate intervention to ameliorate insulin resistance, most women develop an inherent glucose intolerance from altered beta-cell function (7,8) which is not directly associated neither with an increased body mass index (BMI) or age (9). PCOS has been described as an independent risk factor for dyslipidaemia and hypertension (10,11), resulting in overall higher prevalence of women affected also with metabolic syndrome (12). A strong association has been proven between hyperinsulinemia and hyperandrogenism, however the exact mechanism behind this dysfunctional relationship in PCOS remains unclear (13). Indeed, the etiology of this multifactorial syndrome after decades of research still eludes us, its complexity being defined both by a genetic and epigenetic susceptibility, dysfunction in both hypothalamic and ovarian regions translating into excessive circulating androgens, and insulin resistance aggravating adipose tissue function and inflammation (1). Furthermore, the diagnosis itself of PCOS remains one of exclusion, with assessment and management being often inconsistent and as a result, up to 70% of affected women remain undiagnosed (14).

A chronic state of underlying inflammation in PCOS is interestingly combined with joint appearance of multiple autoimmune conditions. PCOS is very often diagnosed with an early onset of type 2 diabetes mellitus (T2D) (15), and an increased risk of autoimmune thyroid disease (AITD) as well as hypothyroidism (2). Nonetheless, an autoimmune cause has yet not been identified (16–18) with most research addressing the immediate improvement and clinical management of the metabolic conditions. Recent

findings have observed variations in B cell compartments associated with PCOS, although it remains unclear whether these observed altered immune frequencies are a cause or a consequence of the hyperandrogenic hormonal milieu.

Hence, to put the results of this dissertation into perspective, the introduction will elaborate on both the pathophysiology of PCOS and the key role of chronic inflammation, as well as the notion of immune cells exacerbating metabolic disease, with the aim to briefly review all literature regarding inflammatory mediators in PCOS and downstream effects with an emphasis on metabolic features.

1.1.2 Definition and diagnosis of PCOS

PCOS is a multifactorial condition which in its complexity includes a variety of clinical manifestations although the main accepted characteristics are clinical or biochemical hyperandrogenism, ovulatory dysfunction (oligo- or anovulation) and a polycystic ovarian morphology, in the absence of any of the following similar diseases: Cushing syndrome, congenital adrenal hyperplasia, androgen-producing tumors or hyperprolactinemia among others. Understanding the difficulties for a proper diagnosis of the syndrome are crucial in explaining why research has struggled so long in identifying molecular targets to improve adverse events on adult health.

As most literature recall, the notion of *polycystic ovaries* were notably first described in 1844 by Chereau and Rokitansky based on accumulated growth-arrested follicles found in the ovaries of affected women (19). However, it wasn't until 1935 that the heterogeneity of this multifactorial condition became more evident by the famously traced description of Dr.s Stein and Leventhal of seven women presenting multiple associated features such as amenorrhea, three women being also obese, four suffering from hirsutism (among which one woman also overweight), and one strongly acneic (19). Since then, PCOS has come to be described as possibly the most widespread endocrine-metabolic condition among women worldwide. It remains however clinically heterogeneous, with no single diagnostic test available to this date.

Lack of an exact etiology has historically been due to the absence of a clear and agreed upon outline of the syndrome and its phenotypical limits, so that the diagnostic features have equally evolved in time varying the prevalence between such a wide range of 5–

18%. The general diagnostic criteria were first proposed by the National Institute of Health in 1990, five decades following the earliest characterization of the syndrome (NIH 1990 criteria). The NIH criteria emphasized hyperandrogenism and/or hyperandrogenemia as a hallmark feature having a crucial role in PCOS pathophysiology, formally acknowledging it's direct link to hyperinsulinism and disease severity, along with menstrual dysfunction (oligo/anovulation) and following the exclusion of known associated disorders (1).

Ongoing research over the next years led to a more refined definition as proposed in 2003 at the Rotterdam conference (Rotterdam 2003 criteria) by the European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM). The new recommendations stated that PCOS in adult women should rather be diagnosed based on the presence of two out of three features: hyperandrogenism, menstrual disturbance, and multifollicular ovarian morphology (PCOM) (20). It is worth noting that the decision to include a measurement of ovarian dysfunction such as PCOM was further acknowledged in 2006 by the androgen excess and PCOS society (AE-PCOS). The 2003 consensus workshop reinforced the importance of excluding conditions such as thyroid dysfunction, hyperprolactinaemia, non-classical congenital hyperplasia biochemically; as well as other conditions such as Cushing's syndrome if indicated clinically. These diagnostic criteria generated four main phenotypes with significant clinical heterogeneity and individual phenotypes with or without obesity, that typically arise in puberty and may evolve throughout the different stages of adolescent and adult life (21).

Recommendations for a diagnosis of PCOS suggest a clinical assessment based on irregular menstrual cycles and clinical hyperandrogenism, accompanied by proper exclusion of other causes. Indeed, this adjusted model would allow to evaluate most of the necessary criteria, excluding ovarian morphology, solely by a detailed patient history and physical examination. Irregular menstrual cycles frequency appearances have been defined as less than 21 or more than 35 days assessed 3 years after the first menstrual period until perimenopause or less than 8 cycles each year. Prior to this stage in a woman's life, irregular cycles may be generally the case due to pubertal transition, however, should not exceed 45 days already after the first year of menarche (14,22,23). Regular cycles do not completely exclude ovulatory dysfunction, therefore

PCOM may be required when only one between oligomenorrhoea or hyperandrogenism features are recognized. The refinement of this criteria reduced potential over diagnosis particularly in regard to adolescents. This improvement was also due to a standardized threshold for PCOM, based on the usage of a transducer and transvaginal ultrasound which decreased the prevalence among adolescents from 30% to 16% at 14–16 years of age (23). A new approach is therefore the measurement of anti-Muellerian hormone (AMH) to have a positively correlated orientation about PCOM until menopause (LIT) (24–26). AMH is a dimeric glycoprotein of the transforming growth factor-beta (TGF-β) superfamily, released mainly by the granulosa cells within the ovary, which has been shown to increase 2- to 4-fold in serum and follicular fluid of women with PCOS due to both the elevated number of undeveloped small antral follicles and an overexpression of AMH and AMHR2 by their granulosa cells (27). AMH acts as a paracrine inhibitor during follicular cellular differentiation by reducing the availability of the follicle-stimulating hormone (FSH)-stimulated aromatase expression (28), and reduces the follicle sensitivity to FSH by impacting the FSH mRNA expression (29,30).

In normo-ovulatory women, AMH does however respond negatively to the early stage increase of estradiol allowing for follicular growth. Both testosterone and dehydroepiandrosterone are commonly aromatized to estrogens upon promoting basal follicle growth, so there are no direct correlations suggesting any AR activation effects on AMH levels. However, high or supraphysiological androgen concentrations such as in patients with PCOS have been positively associated with AMH levels by several research teams (31,32) and the effects may be related to unique reactions within their specific hormonal setting, such as the LH induced up-regulation of both AMH mRNA and protein expression in human luteinized granulosa cells which does not occur in normo-ovulatory women (30,33) as well as the disrupted estrogen receptor ESR1/ESR2 ratio in granulosa cells which may be preventing all inhibitory effect of estrogens on AMH expression in PCOS (34). Coinciding with the inhibitory action of AMH on the process of follicle selection, indeed compared to ovulatory women with PCOS, anovulatory women with the same condition may present AMH concentrations up to 18 times higher (35).

In the clinics, AMH has been adopted as a valid marker to estimate the pool of residual primordial follicles (36) as serum levels are generally quite stable during menstrual cycles, rather gradually decreasing with age. The assessment of clinical hyperandrogenism is based on the presence of acne, androgenic alopecia, and excess body and/or facial hair growth or hirsutism. The latter is based on a standardized visual scoring system, the modified Ferriman-Gallwey score (mFG), defined by excess terminal hair (indicated by a length exceeding 5 mm) in nine androgenic dependent areas, with scores of more than 4–6 indicative of hirsutism. While prevalence of hirsutism appears to remain homogenous across ethnicities, severity may vary hence the variance in the mFG cut-off scores range (37).

Biochemical hyperandrogenism may be assessed based on serum testosterone (T) concentrations and taking into account it's clearance and bioavailability may allow to determine the most suitable measurement. In circulation, testosterone can be bound either to albumin or to the binding protein sex hormone-binding globulin (SHBG), while approximately 1% in women is present in the free, nonprotein state (38). The bioactive portion of testosterone consists of the free fraction plus the albumin-bound fraction, excluding testosterone bound to SHBG. SHBG levels can fluctuate, often increasing with the use of estrogen-containing oral contraceptives, hyperthyroidism, or weight loss, and decreasing in cases of hypothyroidism, obesity, or elevated androgens. These variations in SHBG concentration directly affect the overall total testosterone levels. The bioavailable testosterone (BioT) measurement excludes SHBG-bound component taking into account only the bioactive form, and the free androgen index (FAI) is a calculated measure of testosterone adjusted for abnormalities in SHBG, they are presumed to be a more reliable index of measurement for hyperandrogenism and are then the measurement of total testosterone concentration alone, assessed preferably by liquid chromatography–mass spectrometry (LC-MS) or by high-quality assays (39). While total testosterone is often modestly elevated or even within the upper limit of normal, free testosterone and FAI are usually elevated (14,20). However, in the case neither values meet a clear cut off, the circulating levels of androstenedione (A4), an intermediate in the biosynthesis of testosterone, and dehydroepiandrosterone sulfate (DHEAS) may be considered. These act as pre-hormones. Hepatic conversion of A4 results in T, a pathway that is particularly important in normoandrogenic women

as it accounts for 50–60% of circulating T. While A4 is produced in large amounts by the ovaries (50%), DHEAS originates mainly from the adrenal cortices and is a stable marker for excess adrenal steroidogenesis or adrenal precursor androgen excess (APA) (40). However, it is to be noted that generic cut-off values may also be imprecise, hence the androgen levels defined for value interpretation are recommended to be based on reference ranges specifically for the laboratory in question, as high variability has been registered among assays, methods, and various laboratories.

To recognize a PCOS-derived hyperandrogenic state, principal disorders that need to be excluded are steroid 21-hydroxylase (CYP21A2) deficient non-classic adrenal hyperplasia (NCAH), hyperprolactinemia and thyroid dysfunction, e.g. hypothyroidism. Clinical evaluation should also asses for androgen-secreting neoplasms, as well as the use of androgenic drugs.

Overall, these criteria enabled a phenotypic classification that enhances both a more precise research and better clinical practice in managing patient conditions based on their risk of co-morbidities (20).

In brief, phenotypes considered *classic* PCOS state are the Phenotype “A”: affected by hyperandrogenism, ovulatory dysfunction and PCOM; phenotype “B”: hyperandrogenism plus ovulatory dysfunction. These represent two thirds of cases, with metabolic features (impaired glucose tolerance, metabolic syndrome, and commonly type 2 diabetes) and increased BMI being aggravating factors (41). Phenotype “C” or *ovulatory* PCOS state, is a combination of hyperandrogenism plus PCOM. Insulin resistance is generally reduced and BMI can often be within normal ranges. Finally, phenotype “D” or the *normoandrogenic* PCOS which includes ovulatory dysfunction plus PCOM which is generally defined by normal serum androgens and no clinical hyperandrogenism.

Lastly, it remains vital to mention how the development of a diagnostic method may also have been hindered by the presence of overarching difficulties that to this day affect gender-based research and research on women's health in particular (42). A Swedish study already in 1997-1999 noted such reasons for the exclusion of women from clinical trials being described as lack of sufficient physiological data, availability of repeated studies as previous investigations used only men so as to obtain

comparable data, and the economic costs of research in women (43). In fact, guideline recommendations would suggest including women in different hormonal states (44–46) however the economic effect of such a “gold standard” methodology may lead to potentially quadrupling the budgets in research grants with immediate repercussions on their future development.

1.1.3 Epidemiology

The prevalence of PCOS has varied in history, in part depending also on the diagnostic criteria in use. In addition, the differences in socioeconomic level, medical care access, prevalence of influential risk factors, health and education/awareness are also among the possible causes of substantial geographic disparities in the prevalence rate (47). It is important to briefly discuss these motifs, as they remain an important area of research, possibly stressing the need for new revisions of the relevant definitions and diagnostic clinical models.

By adopting the initial diagnostic criteria recommended by the 1990 NIH proceedings, noted PCOS prevalence’s in a sampled population ranged initially between 6.1% and 8.7%. The newly updated Rotterdam criteria had the effect of increasing prevalence rates to higher scores ranging between 17.8% and 19.9% in the same sampled population (4,48). As mentioned in the previous section, this underlines the importance phenotypic classification and sample heterogeneity which can only be properly described with the adequate diagnostic models. A meta-analysis review reported the overall prevalence rates of PCOS according to Rotterdam and AE-PCOS Society criteria to be similar, while they were twice as high as those according to NIH criteria (47). Additionally, prevalence of PCOS was quite heterogeneous across geographical regions. For instance, the lowest rates for oligo-anovulation were in Europe, while hirsutism and hyperandrogenemia were found to be uncommon in Asia. The disparity in these results can be attributed in part to the lack of agreed upon phenotype definitions. In this regard, the Rotterdam criteria seemed to favour the diagnosis of PCOS particularly in ethnic groups who for instance do not present clear features of clinical hyperandrogenism. For these reasons, it remains relevant to consider potential local usage of more specific diagnostic criteria as well as the adoption of treatment approaches according to geographical region. In addition, it underscores the need for

future research to explore the use of other objective biochemical diagnostic findings, such as anti-Müllerian hormone measurements along with inflammatory biomarkers, both for translational and clinical applications.

1.2 Pathophysiology

1.2.1 Gonadotropin abnormalities and polycystic ovaries

From an early stage, multiple evidence pinpoint hyperandrogenism as the main driving factor characterizing PCO syndrome. Women with PCOS, particularly during reproductive age, have reported circulating levels of androgens threefold higher compared to age matched controls, values which are directly associated with disease severity particularly with complications affecting reproductive and metabolic function (49). Ovarian hypersecretion is the major origin of circulating androgens in PCOS, as well as the most heritable phenotypic PCOS trait (50). However, it is important to note that among women with clinical hyperandrogenism, only 80–85% are confirmed to be affected with PCOS (3,51), and acne and diffuse alopecia may be even less distinctive. Moreover, as briefly mentioned previously, interpretation of hyperandrogenemia may strongly depend on the assays used as well as the normative values of the control population (52,53).

Although both the definition and the diagnostic criteria for PCOS majorly highlight an ovarian dysfunction, the syndrome does comprise several different etiologies which certainly have a prominent effect on the overall pathophysiology.

First of all, the intrinsic ovarian hypersecretion seems to be driven in part by the action of gonadotropin releasing hormone (GnRH) neurons (54). GnRH pulses, specifically the high and low frequency, support the production of either luteinizing hormone (LH) or follicle-stimulating hormone (FSH) respectively. This stimulus initiates the main signalling pathway allowing central control of the reproductive system. Indeed a tight regulation between the hypothalamic–pituitary–gonadal (HPG) axis and positive and negative feedback loops orchestrate the production and release of LH and FSH in parallel with follicular development (55). FSH levels increase in the early follicular

phase, which triggers secretion of estradiol (E2) which is the main hormone involved in the feedback loops regulating the HPG axis, together with progesterone and inhibin. E2 favours a rapid GnRH pulsatility and selectively inhibits FSH release during the late follicular phase (56). The accelerated GnRH pulsatility induces an increase in circulating LH, which further stimulates E2 secretion. This increase in E2 reaches a mid-menstrual cycle threshold creating a temporary surge of both LH and FSH connected to ovulation. At this point, luteinization of the ruptured follicle allows for the secretion of progesterone, which acts as a negative feedback on the hypothalamus reducing the GnRH pulse frequency leading to the next cycle through FSH secretion increase (56). It is often in synchrony with the pubertal increase in LH secretion that PCOS symptoms first occur and women with PCOS exhibit a two to three fold elevation in circulating LH versus FSH levels due to persistently high GnRH pulse amplitude and frequency (57,58) which favours increased LH and relative FSH deficiency (59). Dose-response protocols have shown that such elevated circulating LH in part reflects a diminished sensitivity of the hypothalamic GnRH pulse generator to suppression by E2 and progesterone, an abnormality in the negative regulation of hypothalamic GnRH secretion which may partially be impaired in PCOS (60). This neuroendocrine irregularity directly contributes to ovarian excess androgen levels and ovulatory dysfunction. Interestingly, the hypothalamic resistance to sex steroid negative feedback appears to be due for the most part to the state of hyperandrogenemia *per se*, as it has been shown to reverse with the introduction of an androgen-receptor antagonist such as flutamide (61). How variations in the secretion of LH affect circulating androgens has been investigated in studies using GnRH agonists which have proven to markedly reduce androgen production (62). These protocols based on desensitization of the LH release decreased circulating LH as well as the measured levels of serum androstenedione and testosterone. All together, these results describe a vicious cycle based on excessive circulating androgens which reduce hypothalamic feedback inhibition allowing for pubertal increase in GnRH pulsatility; hence a gonadotropin dysfunction which then further promotes additional hyperandrogenemia and an on-going ovulatory disturbance.

The synergistic action of FSH and LH is required both for follicular growth (55) and in order to activate progesterone and E2 feedback by the granulosa lutein cells (63).

During ovarian follicular development, gonadotropin-independent primordial follicles further develop into primary and secondary follicles, which express FSH and LH receptors among which one large dominant antral follicle is selected to ovulate. The steps eventually leading to ovulation require both balanced and coordinated interactions between reproductive, metabolic and intra-ovarian factors. Abnormalities in LH:FSH ratio resulting in long-lasting increased LH and decreased circulating FSH are particularly evident among lean women with PCOS, as obesity tends to lower LH pulse amplitude (64–67). This hormonal imbalance results in abnormal follicular maturation as both pre-antral and antral follicles inevitably accumulate and cannot develop properly (68). The interruption of follicular development increases granulosa-cell production of AMH, with increases in circulating values up to three fold (68). This is particularly of relevance as increasing evidence demonstrates how AMH plays a role in neuroendocrine control of reproduction and in gonadotropin action, directly stimulating GnRH neuron activity, increasing LH pulsatility and secretion (69,70). AMH receptors were found in hypothalamic GnRH neurons and in gonadotrope-derived cell lines (71,72). In line with these findings, daughters of women with PCOS, who face an elevated risk of developing the syndrome (49), exhibit higher circulating LH and AMH concentrations, and are positively correlated (73). This overproduction of AMH by the granulosa cells may antagonize FSH activity in the follicles (74). In fact, AMH inhibits follicle growth by decreasing the sensitivity of ovarian follicles to FSH (75). Follicular arrest is therefore accompanied by abnormal menstrual bleeding, anovulatory infertility and enlarged polycystic ovaries characterised by the accumulation of antral follicles from which originates the polycystic morphology (76) and is now increasingly used in clinical diagnosis of PCOM, when ovarian ultrasound is not available.

1.2.2 Androgen and estrogen activation in PCOS

Both androgens and estrogens are involved in regulating the healthy course of female reproductive function (77,78). Folliculogenesis highly depends on a balanced androgen production and aromatase conversion into estrogen within the theca interna and granulosa cells of the mature ovarian follicles (79,80). Increasing estrogens and an overall shift from androgenic to an estrogenic follicular microenvironment induce a negative feedback signal to the HPG axis decreasing levels of FSH, which allow for

selection of a large dominant follicle (81). At this point, briefly introducing the mechanism of androgen synthesis both in ovaries and adrenals may be helpful to underscore the alterations in the androgen receptor activation pathway which are affected in PCOS.

In both organs, steroid hormones biosynthesis begins from cholesterol side-chain cleavage as the first catalytic step in order to allow cholesterol to pregnenolone (PE) conversion (82). Steroid-producing cells contain the necessary cytochrome P450 enzymes for this process such as side-chain cleavage (P450-SCC) and 17α-hydroxylase (17α-OH), which act both as a 17-hydroxylase and as a 17,20-lyase. Estradiol biosynthesis through cytochrome P450c17α (CYP17) enzymes is catalysed via two metabolic pathways, the Δ5 or the Δ4-pathway utilising steroid substrate pregnenolone and progesterone, respectively. Cytochrome affinity for either steroid substrate is quite similar, however the predominant pathway is via the 17 OH pregnenolone (Δ5 substrate). Here the 17-hydroxylase enzyme promotes rapid conversion of pregnenolone into 17-hydroxypregnenolone (17-OHPE). Acting in tandem, although at a slower rate, the 17,20-lyase enzyme converts 17-OHPE into dehydroepiandrosterone (DHEA). All while via the Δ4-pathway 17-hydroxylase enzyme catalyses progesterone into 17-hydroxyprogesterone (17-OHP4) which is further converted by the 17,20-lyase enzyme into androstenedione (A4). However, in humans progesterone remains a disadvantageous source for androgen biosynthesis as the conversion of 17α hydroxyprogesterone to androstenedione is generally less efficient (83,84).

Compared to healthy individuals, greater activity of 17,20-lyase has been demonstrated in PCOS patients (85). Clearly androstenedione is a key intermediary steroid that can either convert to Testosterone which would typically undergo a final aromatase conversion to estradiol or alternatively, androstenedione may also be aromatized to estrone (E1), from which E2 is produced by the 17β-hydroxysteroid dehydrogenase (17β-HSD). E2 is relevant as the main biologically active form among estrogens (86). In healthy premenopausal women, ovaries are the primary site of E2 production. It is also synthesized in a number of extragonadal tissues through the conversion of testosterone by cytochrome p450 aromatase, including bone, breast, adipose tissue, and the brain (87).

Accumulated androgen metabolites which convert into estrogens and androstanes (3β- Androstanediol) are ultimately direct ligands of the estrogen receptor (ER). Therefore, aside from direct androgenic actions, PCOS traits could derive from an indirect or even dysfunctional activation of the ER (84). The activation of sex steroid receptors involving ligand-induced transcription factors are highly coordinated processes which trans activate target genes ligand-dependently (84). Androgens exert their effects mainly through the androgen receptor (AR), while estrogens act through the estrogen receptors (ER), which exist in two forms: ERα and ERβ. Another estrogen receptor, originally identified from human and rat tissues is the G protein-coupled estrogen receptor (GPER). This intracellular transmembrane estrogen receptor is widely found in numerous human tissues despite having a reduced estrogen-binding affinity compared to other estrogen receptors. However, it can induce a rapid estrogen-mediated activation of ERK1/2 (88). During normal menstrual cycles, the endometrium undergoes rapid growth in response to estrogen peak, particularly of E2, in the follicular phase increasing endometrial sensitivity through higher ERα levels. Conversely, it is in the luteal phase that the corpus luteum reduces circulating estrogen rather secreting progesterone to provide an appropriate uterine environment for maintaining pregnancy. Early studies already noted how women with PCOS commonly present estradiol values that remain within the early to mid-follicular range lacking the expected mid-cycle increases (89), whereas E1 levels are increased, resulting in a shift of the E1/E2 ratio (90). This is in line with higher activity of aromatase enzymes and 17-ß hydroxysteroid dehydrogenase shown in cumulated peripheral fat cells of PCOS women leading to peripheral aromatization of circulating androstenedione levels (91) which triggers an increase in serum E1 values. Another cycling element which does not seem to respond to regulating feedback, are the ER pathways in PCOS endometrium. During a normal ovulatory cycle, ER and progesterone receptor expression become down-regulated in the secretory phase (92). Endometrial ER expression has been described to persist in women with PCOS well into the secretory phase and is high in both stroma and luminal epithelium (93). Interestingly, ERα/ERβ ratio in PCOS patients are higher compared to control groups (94). This may lead to inappropriate ER responses, with consequently higher risk for pregnancy loss and susceptibility to hyperplasia and endometrial cancer (95).

Testosterone and DHT are the only androgens that can actively bind to the AR, whereas the precursor androgens, dehydroepiandrosterone (DHEA) and androstenedione (A4), must be further converted to produce androgenic effects (84). Recent studies have identified a direct action of DHEA in the brain and vascular endothelial cells through its sulphated form, DHEA sulfate (DHEAS) (96,97). This metabolite has been found to influence neuronal excitability by modulating neurotransmitter receptors, such as GABA and N-methyl-D-aspartate (NMDA) receptors (98). This finding is particularly relevant given emerging evidence highlighting altered GABAergic stimulus to GnRH neurons as a possible key process in the development of PCOS (99).

Preclinical studies with PCOS animal models have been vital both in corroborating the wide range of PCOS-like characteristics induced by exposure to testosterone or DHT (100), as well as attempting to clarify individual pathways stemming from either AR or ER activation in PCOS. Findings based on PCOS animal models support a pivotal role for AR activation in the development of PCOS and will be thoroughly discussed in a following section of this dissertation. By utilizing an AR antagonist such as flutamide, key PCOS features linked to its activation have been isolated both in hyperandrogenised mice and sheep (101). Indeed the same reproductive complications experienced in human PCOS can be replicated through in utero exposure of DHT of adult wildtype mice, but not in the case of global AR knockout (ARKO) mice (102).

Moreover, a recent study attempting late-gestation exposure of DHT in utero demonstrated the direct heritability of PCOS-like phenotypes in female offspring, both F1 and F3 (49), which altogether suggest that excessive AR activation in the prenatal stage may strongly predispose to the development of PCOS, with potential transgenerational effects in female offspring in their adult life. It is worth noting that selective silencing of AR signalling in brain tissue of peripubertal DHT induced PCOS mouse model seems to protect against the development of the majority of PCOS traits, and further research on AR-mediated neuroendocrine mechanisms remains incredibly relevant (103). Finally, experimental animal studies also support the notion that estrogen receptor activation pathways contribute to the development of PCOS features. Notably, research using PCOS sheep model has enhanced our

understanding of how adult disease programming is indeed influenced by imbalanced activation of both pathways, androgenic and estrogenic (104,105).

1.2.3 Insulin resistance and adipose tissue dysfunction

Insulin resistance (IR) is a common condition in many women with PCOS, often occurring independently of predictions based solely on their BMI. (6). This is due to abnormal insulin signalling combined with dysfunctional tissue responsiveness, further aggravated by obesity. Euglycaemic–hyperinsulinaemic clamps studies suggest an often overlooked occurrence ranging between 75–95% of both IR and associated hyperinsulinemia affecting women diagnosed with PCOS (106). This results in increased activity of the ovarian cytochrome P450 17A1 and its hydroxylase activity in the theca cells, which aggravates folliculogenesis by increasing ovarian androgen concentrations (107). The increased androgen production is due to hyperinsulinemia and its main effector molecule insulin-like growth factor 1 (IGF-1). The resulting diminished circulating levels of hepatic sex hormone-binding globulin as well as IGF-binding protein production, increase serum IGF-1 bioactivity and higher free testosterone levels (108,109).

While the concept of PCOS-associated (intrinsic) IR may have been initially disputed because of inconsistent results, evidence of unique PCOS - related insulin signalling abnormalities have since been proven by an increasing number of mechanistic studies (110,111). Hyperandrogenism in women is per se an independent risk factor of developing insulin resistance compared to phenotypes without hyperandrogenism and healthy controls (112). Indeed, higher circulating androgens are associated with developing IR also in women with no other Rotterdam criterion and should be considered for screening to avoid later metabolic risks. This represents a substantial health burden which is largely overlooked. Meta-analysis suggests an underlying increased risk for women affected with PCOS up to 4.43-fold (OR, 95% CI 4.06 – 4.82) of incurring type 2 diabetes (T2D), even following corrections for BMI (113). Understanding the relevance of IR within the specific hormonal setting of PCOS has allowed for better diagnostic criteria and improved personalised therapies.

Following the Rotterdam criteria and the inclusion of women with more moderate reproductive and metabolic conditions, euglycaemic–hyperinsulinemic clamps are

recommended as the gold standard for research-based assessment of IR and may be a more accurate reflection of IR than the commonly utilized HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) scores (106). The homeostatic model assessment is a simple and widely used method for determining insulin resistance based on an equation calculated multiplying fasting plasma insulin (FPI) by fasting plasma glucose (FPG), then dividing by the constant 22.5 (114). However, an issue that arises is determining the insulin resistance cut-off point, hence the lack of standardized reference range may hinder its application (115). Based on the gold standard clamp technique, including an age-appropriate control group to define IR cut-offs in healthy controls as recommended (116), a recent observational study noted that 85% of women with PCOS showed IR with 75% of lean women representing a milder reproductive PCOS phenotype, and 95% of obese women having WHO-defined IR (106). These findings emphasize the importance of further research as potential genetic basis for PCOS-associated IR mechanisms remain unclear. Currently used clinical markers for IR are an increased waist circumference associated with expanded adipocytes (117) even in normal-weight women (118).

These percentages certainly are of concern and the importance of clinical recommendations for lifestyle interventions remain a priority as preventive means to decrease the extrinsic IR inflammation.

In regard to BMI, the impact of obesity is a well-accepted independent factor of extrinsic IR in the general population (119). Obesity itself has numerous endocrine effects such as increased adipose androgen production (110), suppressed sex hormone-binding globulin (SHBG) (120), progressive IR with metabolic consequences and higher peripheral values of androgen exposure.

Adiposity-dependent IR is inevitably co-occurring with PCOS, as the age adjusted prevalence of obesity among women diagnosed with PCOS is notably higher than matching controls (121). Higher values of aldoketo reductase type 3 (AKR1C3) have been described among women with PCOS which may suggest an insulin driven production of testosterone by the adipose tissue leading to tissue insulin resistance (122). The debate remains controversial however, as when investigating the contribution of visceral fat to IR, differences in insulin sensitivity registered among

cohorts may not proportional to the degree of adiposity (123) and could more likely be a contributor to extrinsic IR (106).

Other mechanisms of intrinsic PCOS IR are primary defects in insulin-mediated glucose transport which affect both skeletal muscle and adipose tissue (123,124), altered transmembrane protein production (GLUT4) (125,126) and insulin or adrenergic regulated lipolysis (127,128) in adipocytes (fibroblasts), despite normal insulin binding. Moreover, insulin signalling may be disrupted at the intracellular level in fibroblasts, adipocytes, and myocytes in some cases due to aberrant autophosphorylation of the insulin receptor or altered phosphorylation of insulin receptor substrate, glycogen synthase kinase 3, or the serine/threonine-protein kinase AKT (123,125,129,130). Hyperinsulinemia is associated with overall increased weight, and more specifically inhibiting lipolysis in adipocytes with a propensity for lipogenesis (131). However interestingly in PCOS, insulin resistance might result from a rather defective lipogenic response of adipose tissue to properly enlarge (132–134).

In conclusion, further research is most certainly warranted as the adipose tissue is a metabolically dynamic organ, secreting numerous factors regulating energy expenditure and food intake, supporting integrative neurocircuits for metabolism and inflammation. Continuous crosstalk occurs between adipocytes and the immune system, as this tissue is regularly infiltrated generating complex paracrine interactions (135).

1.2.4 Heritability of PCOS, genetics and intrauterine environment

Cumulative evidence does suggest that an adverse maternal–fetal environment might potentially predispose offspring to develop reproductive, metabolic, and psychological disorders, independently from genetic inheritance and sex (136–138). PCOS is an independent risk factor for perinatal complications with hyperandrogenism being accompanied by higher insulin and AMH values (139,140), often aggravated by excessive gestational weight gain compared to ovulatory controls (141). Other PCOS associated obstetrical and cardio-metabolic complications include gestational diabetes, pre-eclampsia, preterm delivery and small or large offspring size for gestational age (142–144). A presumed decisive interval for fetal androgen excess has

been suggested, specifically during which developmental programming occurs (145). Indeed, despite clinical studies not yet being able to define causal conclusions based on exact gestational weeks, in most studies daughters of women with PCOS demonstrated the presence of a distinct marker of in utero androgen exposure, such as elongated anogenital distance (146). Further studies based on different developmental stages, identified among daughters higher circulating AMH, and similarly higher testosterone found in fetuses of women with PCOS compared to fetuses from non-PCOS mothers, irrespective of gender (147). During the postmenarchal period instead, three main pathophysiologic components were strongly associated with being a daughter of a women affected with PCOS: increased LH secretion (neuroendocrine component), higher androgen levels, and a hyperinsulinemic response to an oral glucose tolerance challenge (73). Finally, a preliminary evidence of heritability based on specific epigenetic PCOS reprogramming signatures was found in a pilot study profiling the methylation of the DNA extracted from umbilical cord blood of anovulatory PCOS women undergoing *in vitro* fertilization (148).

Transgenerational studies remain inevitably challenging. Register-based and clinical case–control studies allow for likelihood analysis of developing PCOS in first-generation offspring, however causal associations or confounding genetic factors remain difficult to isolate. Longitudinal human cohort studies are equally demanding, as often registers are not sufficiently updated and daughters may be too young for second and third generation analysis (49). Studies based on animal models with female mouse offspring have confirmed heritability of reproductive and metabolic PCOS traits, which do seem to be passed on until the third-generation offspring, although the limitations of these studies need to be considered (101) and these results have yet to be confirmed in humans. Transgenerational transmission was attained following prenatal dihydrotestosterone (49) or AMH exposure (149). In regard to potential PCOS-epigenetic signatures carried over by germ cells or somatic cells further transmitting PCOS throughout generations, both transcriptional and mitochondrial perturbations of oocytes (49) and ovarian DNA methylation (149) have been described.

That PCOS runs in families is a commonly accepted notion, nonetheless the number of recognized susceptibility loci accountable for potentially developing PCOS-like traits (currently ~20) (150) remains rather low considering the estimated heritability of 70% based on phenotypic and family aggregation studies (151–155). A general agreement on mode and pattern of inheritance has not been acknowledged.

Progress in identifying heritable genetic components seems to be limited when compared with our understanding of the genetic influence in other equally common disorders such as T2DM. Among predisposing genes and mutations based on association and linkage studies with functional candidate genes, few exhibited significant evidence of association with PCOS (156). These results are somewhat not surprising, as PCOS is considered not to have a monogenic basis and considering the aforementioned influence of the renewed Rotterdam diagnostic criteria which identified multiple different phenotypes, hence genes with a major phenotypic impact are possibly not expected to be responsible for PCOS (156). The lack of progress has clearly multiple reasons which reflect the difficulties that arise when analysing complex diseases which require robust sample sizes along with the possibility to replicate potential associations in independent studies and cohorts. For this reason, it has been the case of multiple associations being described which unfortunately remain yet to be confirmed.

The largest combined sample of PCOS families studied to date, identified one plausible candidate PCOS susceptibility gene mapping in the interval of chromosome 19p13.2 for resistin (157), a protein hormone released by adipocytes which is central to the development of insulin resistance and insulin action. However, no significant evidence for association with PCOS was found.

Larger case-control studies and genome-wide association studies (GWAS) are generally better suited in describing associations for genetic factors that may influence PCOS reproductive and metabolic phenotypes (158–162). A comprehensive analysis conducting bidirectional mendelian randomization between BMI and PCOS, demonstrated specific PCOS susceptibility loci associated with metabolic PCOS phenotypes (161). In this study genetic risk scores based on BMI-increasing alleles proved how an increase in BMI is causal for PCOS, while being affected with PCOS

does not causally impact BMI, further underlining the influence of obesity on PCOS with important translational implications particularly for patient counselling.

Targeted approaches combined with whole-genome sequencing can also be a strategy in describing lower allele frequency variants which may remain undetected by genome-wide association studies arrays. This has been the case for 37 rare *AMH2* variants (163) and 32 rare *DENND1A* variants (164).

Findings based on genome-wide association studies identified through summary statics a genetic link for the glycaemic traits which connect both type 2 diabetes and PCOS through biological pleiotropy and causal mediation in a BMI-independent manner (165). For this reason, while not being a specific focus of this dissertation it is noteworthy to mention that indeed men with high polygenic risk scores for PCOS have an equally higher risk of developing type 2 diabetes and cardiovascular complications (166,167) along with male pattern baldness (168).

1.3 Clinical features

The complex pathophysiology of this multifactorial condition mirrors the heterogeneous clinical features which appear with different degree both among women affected by PCOS as much as in the different life stages of a single individual. Stemming from the diagnostic traits, reproductive features such as menstrual irregularity, anovulation, hirsutism, infertility (169), and pregnancy complications (142) are among the most common that have already been mentioned.

It is an aim of this thesis to examine and highlight particularly how low-grade inflammation in PCOS, independently of the presence of obesity (170) is inherently relevant in understanding the intrinsic metabolic dysfunction. Individuals with this condition present key metabolic traits such as IR (106), obesity (171), dyslipidaemia (172) and frequent joint appearance of multiple diseases such as diabetes (173) and immunothyropathies (2). Depression and anxiety are among the associated psychological features that often originate conjunctly with body image concerns and mental health disorders (174,175).

1.3.1 Metabolic features

The effects of hyperinsulinemia are the main driving features defining the metabolic outcomes in PCOS, stemming from IR in skeletal muscle and adipose tissue (176). Most women with PCOS are notably also obese. However, as previously mentioned, PCOS does seem to induce a state of insulin resistance which is both independent and additive to the effects of increased adipose tissue (177,178) leading to an earlier onset and higher burden of T2D. Reports of community incidence of T2D describe frequencies of 4·19 per 1000 population per year among women affected with PCOS in comparison to 1·02 per 1000 population in the control group (173). Hyperandrogenism, impaired glucose metabolism and insulin resistance, each separately affect circulating lipid profiles with interrelated mechanisms that require further explanation.

A tendency for a more centripetal and visceral body fat distribution is a typical effect of hyperandrogenism, which favours an increased waist-to-hip ratio, an indication of higher predisposition to develop dyslipidaemia due to increased lipolysis. This insulin-resistant behaviour occurs mainly in central fat where fatty acids are recycled more rapidly (179–181), independently of overall BMI (182).

How hyperinsulinemia perpetuates adipose tissue dysfunction is both a matter of altered adipocyte size combined with adipocyte lipogenesis (131), decreased circulating levels of adiponectin (117), general weight gain (171), and increased circulating leptin levels accompanied by leptin resistance (183). The adipokine leptin typically exhibits circulating levels that rise in proportion to white adipose tissue (WAT) mass under normal physiological conditions. It plays a central role in regulating energy balance by signalling the brain to reduce food intake and increase energy expenditure. Women with PCOS, like individuals with obesity, exhibit elevated levels of leptin in their bloodstream. However, due to the development of leptin resistance in the hypothalamus, these higher leptin levels do not trigger the typical responses associated with elevated leptin, such as enhanced energy expenditure, less appetite, and lower body weight. Leptin is crucial in the overall pathophysiological mechanism and even partial reductions in plasma levels have proven to restore hypothalamic sensitivity with significant improvements both in body weight and IR (184). Leptin

production is modulated by sex hormones—being suppressed by testosterone and elevated by estrogen and progesterone (185)—as well as by numerous inflammatory molecules, which contribute to its broad impact throughout the body. When increased, leptin aggravates the state of hyperandrogenemia as it inhibits aromatase activity, and it's previously described role in the conversion of androgens to estradiol (186). Interestingly, the leptin receptor (LEPR) is widely distributed across various tissues and is also present on most immune cell surfaces, highlighting its role in bridging neuroendocrine and immune system functions (187).

Within the innate immune system, leptin is crucial for normal NK cell function, though chronic high levels of leptin may lead to immune suppression and reduced NK cell proliferation, likely due to leptin resistance(188). Leptin also stimulates monocyte activation and proliferation, which promotes the release of important inflammatory cytokines like IL-1, TNF, and IL-6 (189). These effects vary based on tissue type and receptor form (whether membrane-bound or soluble) and can be either pro- or anti-inflammatory. Interestingly, Acedo and colleagues were able to demonstrate that leptin exposure may result in infiltrating macrophages with very unique properties: indeed leptin induces M2-phenotype surface markers with an M1-typical cytokine producing potential (190). In terms of adaptive immunity, leptin enhances T cell activity, skewing T-helper cells toward a TH1-dominant, proinflammatory response (characterized by IFNγ production) and increasing TH17 cell activity(191). Finally, leptin activates B lymphocytes, prompting the secretion of both proinflammatory cytokines (such as TNF and IL-6) and the regulatory cytokine IL-10 through JAK–STAT and p38MAPK–ERK1/2 pathways (192). In order to understand how adipocyte hypertrophy is relevant to systemic IR it is relevant to discuss both the role and cellular composition of this tissue being a complex metabolic, endocrine and immune organ. With the main function of storing triglycerides as endogenous fuel, the adipose tissue supports the activity of both the liver and skeletal muscle in promoting insulin-stimulated glucose uptake. There are two principal types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). Research indicates that PCOS disrupts basic regulatory processes within WAT, such as lipolysis, vascularization and its distribution and structure (193). In humans WAT deposits mainly in subcutaneous and visceral regions, forming aggregates of two main cell types, adipocytes and connective stroma-vascular

fraction. However, among them forming a highly active metabolic tissue are various interacting cells, including pre-adipocytes, fibroblasts, numerous immune cells, endothelial cells, and mesenchymal stem cells. These cells collectively influence both the endocrine and immune system with direct effects on feeding behaviour, energy balance and reproductive health (194). In women with PCOS, hypertrophic obesity has been described as a main response to IR in contrast to hyperplastic obesity, in other words an increase in fat cell size rather than in their numbers (117,195), Studies have shown adipocyte diameter reported as 25% larger in women with PCOS compared to similarly obese women without the condition. Disrupted hypertrophic adipocytes are intrinsically insulin resistant (196) and their state can lead to cell death, triggering an inflammatory response from macrophages, which form crown-like structures surrounding dead adipocytes. This causes a phenotypic switch in macrophages to a more proinflammatory state, from so called “M2” to “M1”, promoting adipose tissue inflammation (197). However, in vitro PCOS-deriving adipocyte cell studies did not prove incapable of sensing circulating insulin (198). Hence, the fundamental cell processes are disrupted by other specific factors, most likely the excessive values of circulating androgen. Of note, there is also evidence of diminished adipocyte insulin receptor phosphorylation, which translates to an impaired GLUT4 translocation, another element within the mechanism affecting insulin-dependent glucose uptake in PCOS (126).

The combination of systemic IR and hyperandrogenemia fuel catecholamine-induced lipolysis within the adipocyte and fatty acids pour into circulation with commonly altered circulating lipid levels (172) which are of course aggravated in the case of higher BMI values (199). The resulting hypertriglyceridemia is in part also a response from the liver to the influx of lipids which are reorganized under the form of circulating very-low-density lipoproteins (VLDL). Lipid ratio is further compromised by the specific hormonal setting which in favouring androgen receptor activation, diminishes the estrogenic induced activity of the low-density lipoprotein receptor (LDLR) and its catabolic discharge of the same LDL lipoproteins (200). However, the clinical significance of this dyslipidaemia has not been fully clarified, as various PCOS-associated patterns of serum lipid levels have been described (9,201–206). Among these, decreases in high-density lipoprotein (HDL), and elevations of triglycerides (TC), VLDL, LDL as well as

qualitative disorders of the LDL have all been described in PCOS (207). Additionally, antiandrogen administration such as flutamide has led to contradictory resulting lipid profiles (8,203) and would need to be further examined. Nevertheless, women with PCOS are at increased risk of hypertension (208) and although the exact prevalence of cardiovascular disease remains vaguely defined among these women, PCOS continues to cluster as a cardiovascular risk factor. Subclinical atherosclerosis is the main concern as inflammatory insulin resistance promotes an atherothrombotic milieu, with elevated proinflammatory mediators, impairing endothelial function and eventually reducing vasoreactivity (209,210).

Another principal function of the adipose tissue is its central role as an endocrine tissue and immune mediator. It secretes soluble peptides like adipocytokines, adipokines, cytokines, chemokines including leptin, adiponectin, and resistin in coordination with the central nervous system and pituitary gland that attune the intensity of immune and inflammatory responses (194,196,211). Among these, adiponectin is an important factor worth mentioning. Adiponectin has both anti-inflammatory and insulin-sensitizing effects (212). Interestingly women with PCOS exhibit lower adiponectin levels, regardless of whether they are overweight, which likely contributes to the inherent insulin resistance so far described (213)

Other proinflammatory elements such as most of the TNF-α, inducible nitric oxide synthase, and IL-6 produced in adipose tissue are actually derived from macrophages which infiltrate the adipose tissue (211). The interaction among these cells is initiated by the same adipocytes which modulate macrophage processes via upregulation of monocyte chemoattractant protein-1 (MCP-1), macrophage migration inhibitory factor, and macrophage inflammatory protein (MIP)-1α and are found at higher levels in obesity (214). However minimal differences in macrophage density have been described in the subcutaneous adipose tissue between women with and without PCOS (117), and even less has been proven regarding specifically immune cell function changes in the adipose tissue of these individuals.

How infiltrating lymphocytes, especially T cells, are involved in the inflammatory phenotype of this tissue has more recently become apparent and will be discussed later in more depth. However, a relevant finding in preclinical studies described a

rearrangement among adipose T cells of the ratio between CD8(+) effector and CD4(+) helper T cells in response to a high-fat diet, which was associated with macrophage infiltration and the onset of insulin resistance, particularly in the more obese animals. (215).

The acute redistribution of adipose tissue towards visceral depots from subcutaneous areas can indirectly affect other insulin target tissues, such as liver and skeletal muscle as circulating lipids eventually increase fat depots also on these tissues and may interrupt proximal steps of insulin signalling pathway through accumulation of intermediates (216–219) such as the phosphorylation/activation of insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinase (PI3K), and Akt (220–222).

Exercise can truly address IR in skeletal muscle stimulating and inducing both the insulin dependent PI3K/AKT signalling pathway as well as the non-insulin dependent AMPK signalling pathway, increasing insulin utilization efficiency (223). PCOS does indeed reduce its action in skeletal muscle, altering insulin-signalling pathways with a diminished efficacy in up taking glucose (224). Pathways initiated by exercise, such as the PI3K/AKT for instance, can support an increased translocation of glucose transporters particularly GLUT4 for higher transport capacity (225). Data suggests that low insulin sensitivity in PCOS may be due to altered activity of upstream mediators in the described pathways, and similarly for IRS-1 and AKT pathway (123).

The skeletal muscle tissue consists of around 600 muscles, an incredibly dynamic tissue, abide to the control of the nervous system (226) and responsible for 75% of whole glucose uptake (227,228). While it is mainly considered for its role in movement, it is clear how its systems greatly affect the individual's energy process and metabolism. Being involved in the amino acids and carbohydrates, the skeletal muscle tissue supports the regulation of whole body glucose homeostasis. Optimal skeletal muscle health throughout life is crucial in preventing the development of metabolic disturbances. Hence the importance of emphasizing exercise and lifestyle interventions for weight management which are both recommended as an initial treatment in PCOS by evidence-based guidelines (14).

Finally, other mechanisms that have been gaining attention are the mitogen-activated protein kinase (MAPK) pathway which also exhibits altered downstream activity,

potentially contributing to the onset of insulin resistance (130). Additionally, skeletal muscle may be compromised due to incoherent mRNA expression of genes involved in the mitochondrial oxidative function, fatty acid metabolism and calcium balance (229). One theory proposed that the insulin resistance observed might stem from impaired mitochondrial respiration (230) altering ATP synthesis although this finding has been debated (231). Myostatin, also a common regulator of metabolism, has been suggested to influence androgen metabolism in skeletal muscle cells (232) although the precise relationship between androgens and insulin sensitivity in skeletal muscle in humans is still uncertain and requires further investigation.

1.3.2 Reproductive features

PCOS is mainly known for the anovulatory infertility affecting women whom suffer of the syndrome (26,233). It remains an independent risk factor for miscarriage and premature birth as PCOS symptoms including elevated blood pressure all throughout the pregnancy - independent of BMI (234), preeclampsia, developing gestational diabetes (235) and uterine blood flow from the uterine artery (139). Initial primate models allowed for a preliminary understanding of the mechanisms controlling nuclear and cytoplasmic maturation of oocytes paving the way for studies on impaired oocyte developmental competence in women affected with PCOS (236).

Each trimester presents its own risk. During the first trimester, clinically measured pathological increases in resistance of the utero-placental vascular circulation should be considered, which may predict hypertensive disorders and placental malperfusion (237). The risk of developing pre-eclampsia remains significantly higher in women with PCOS (238) and pre-existing maternal pathological features should be assed to avoid adverse perinatal outcome (239).

Generally, pregnancy is accompanied by a surge in both circulating estrogen and progesterone levels and a reduction of SHBG in the second trimester. However, it is at this stage that increasingly higher DHEAS titres are often described in women affected by PCOS and the overall androgen FAI scores remain elevated throughout the second trimester (240). During this period, values of insulin secretion to an oral glucose challenge (OGTT) are also increased and metabolic disturbances associated

with a hyperandrogenic phenotype are a true concern for adversely affecting pregnancy outcome (139,241).

With PCOS, the placenta may exhibit more chorionic villitis and intervillositis particularly at early stages of the pregnancy with a proportionally increased severity among women presenting all the diagnostic criteria (242). More macroscopic placentae lesions have been reported at term (243). Indeed, preeclampsia studies described alterations that could potentially supress the vital exchange of nutrients between mother and fetus (242).

A higher BMI increases the risk of poor pregnancy outcome, exacerbating anovulation and infertility in PCOS. Obesity affects uterine receptivity (244) and is an independent risk factor to adverse obstetric outcomes, regardless of prior or due to an excessive weight gain during pregnancy (245).

One of the main reasons is the risk of developing leptin resistance throughout the pregnancy (246) which may occur following diminished receptor mRNA availability without a concurrent surplus of the protein levels (247), and although serum leptin values remain higher among obese women, numbers are often lower when analysed per unit mass of adipose (248).

A similar situation happens in the case of adiponectin, with the risk of incurring in gestational diabetes. A previously mentioned hormone, adiponectin is released by the maternal adipose tissue and potentially also produced by the placenta at term (249,250). Nonetheless, regardless of its origin, circulating adiponectin decreases with pregnancy but even lower values have been described in overweight women (251).

In this regard, an important notion that has radically changed is considering the placenta an absolute protective filter, or a closed system. Numerous examples of exposure to prenatal androgen have demonstrated that this alone, such as in lean women with PCOS, does induce susceptibility to a metabolic (252), reproductive dysfunction (253) as well as behavioural disorders (254), which develop at different stages of growth and may pass on to subsequent generations (49). Signs of divergent development noted in daughters of women with PCOS include enlarged ovarian volume and as they progress through puberty, they may show elevated insulin

responses, triglycerides, androgens and lower SHBG values compared to controls of the same age (252,255). This pattern persists into adulthood, with the possibility of a relatively better reproductive profile (256). Between 28-36% daughters experience a complete PCOS phenotype (257) although values for circulating androgens may vary (258,259) and the relationship based on maternal androgen levels are yet unclear (258). t's important to mention that these steroid measurements were performed using chemiluminescent immunoassays, which have lower sensitivity compared to methods like liquid or gas chromatography-mass spectrometry (260). Finally, the protective effect of CYP19A1 and its enzymatic expression within the placenta in aiding the conversion of maternal testosterone into estrogens (261) was also reconsidered.

While most human studies indicate different levels of associations rather than causality, intergenerational animal studies of maternal androgen excess have demonstrated the harmful effects on offspring. Maternal androgen excess is widely recognized for inducing a PCOS-like condition in rodents, sheep, and non-human primates. These models show that offspring often experience restriction in their intrauterine growth, and in adulthood, they develop PCOS-like characteristics, including behavioural abnormalities (262–266). Animal studies investigating the impact of maternal obesity have revealed disruptions in placental function (267), fetal overgrowth (268), high blood pressure and impaired responses to glucose (269), stress (270), and irregular methylation in oocytes and liver (271). Obesity can profoundly aggravate this condition seeing as there is already a predisposition leading to postnatal metabolic, vascular, and anatomical problems (272,273). The adverse effects of PCOS on pregnancy outcomes, fetal health, and long-term consequences may be partially explained by disruptions in placental steroidogenesis (274–276), which fail to mitigate the already hyperandrogenic environment (261).

1.3.4 Immunological features

Sex steroid hormones profoundly affect the immune system, and understanding these sexual dimorphic effects may provide important direction towards better treatment of immune-dependent disorders. Among PCOS patients, peripheral white blood cells

have been found to be significantly expanded, with higher values both overall and in the proportion of immune cell subgroups, such as lymphocytes, macrophages, and eosinophils, as compared with healthy controls (277). These alterations are clearly exacerbated by higher BMI, as obesity and insulin resistance are both clinical manifestations of PCOS and play a pivotal role in the regulation of ovarian function. Key drivers of chronic inflammation in PCOS are linked to an unhealthy lifestyle, which includes factors such as noxious diets, little exercise and sleep, and unfavourable environmental triggers, which can lead to both fat accumulation and hyperactivation of the immune system (278). These circumstances can independently of the genetic risk strongly increase the rate of metabolic dysregulation and incident cardiovascular events, increasing the susceptibility for other chronic inflammatory diseases (279) including obesity and atherosclerosis but also diabetes (280), gout (281) and rheumatoid arthritis (282,283).

The association between multiple triggers of inflammation and metabolic diseases is well-recognized, yet, whether and how they can provoke long-term reprogramming of immune cells, leading to potentially long-lasting alterations has been clarified quite recently. First records of inflammation being a central mediator of IR were taken by Feingold and Grunfeld in their notes between 1989 and 1991, observing a sudden surge in circulating glucose values after administration cytokine tumor necrosis factor-α (TNF-α), first gathering the notion that cytokine overproduction is directly linked to hyperglycemia (284,285). This is due to the IKK kinase signalling complex and NF-κB cascade, both of which are highly active in a state of obesity and insulin resistance (286,287). Inflammation is instrumental to the development of systemic insulin resistance in all insulin targeted organs such as fat (211,288), liver (289), muscle (290,291), including the ovary (292). Understanding the role of immune cells is key to uncovering both the pathogenesis and treatment for inflammation in PCOS. The following sections will outline the most prominent immune cell types and their relationship to inflammation within the context of PCOS hyperandrogenic hormonal milieu.

1.3.4.1 Macrophages

Macrophages constitute a broad family of tissue-resident, professional phagocytic cells, including brain microglia, liver Kupffer cells, lung alveolar macrophages, and epidermal Langerhans cells, that play an important role not only in defense against pathogens but also in tissue development and homeostasis. In contrast to the long-held view that circulating monocytes constitute the primary source for the replenishment of tissue-resident macrophages throughout life, macrophages broadly consist of two classes: tissue-resident macrophages and infiltrating macrophages. Resident macrophages of most tissues are established prenatally from embryonic precursors and, under homeostatic conditions, are maintained by self-renewal independently of monocytes. (293,294). Considered as part of the innate immune system, macrophages foster specialized responses to local cues with a strong phagocytic capability, thereby promoting tissue differentiation, function, and homeostasis. They are also the most ubiquitous immune cells within the adipose tissue and ovary, (295,296), and are involved in multiple processes, such as folliculogenesis and ovulation (296,297). Their presence in the uterus depends greatly on the various stages of ovarian cycle, with major increases during both progesterone-regulated ovulation and luteal phases (296).

In obesity, as a general model, macrophages have been shown to affect glucose metabolism and systemic insulin resistance (298) with a mechanism revolving around the accumulation of adipose tissue macrophages (ATMs) in a dysfunctional adipose tissue. As mentioned previously, a large part of the cells shaping the adipose tissue are in a great majority infiltrated immune cells and particularly ATMs comprising up to 40% of the cells within an obese adipose tissue (211). In such an environment, activated proinflammatory ATMs release numerous cytokines, among which TNF-α and interleukin-1β (IL-1β) (298) which diminish insulin sensitivity of responsive cells (adipocytes, hepatocytes and myocytes). Among the different fat depots, it is the visceral adipose tissue that recruits' ATMs with a proinflammatory profile the most, from which metabolically more harmful cytokines are released (299). Another relevant aspect is the anatomical distribution into circulation of both cytokines and free fatty acids (FFAs): being the visceral fat directly connected to the hepatic blood duct it is a

major contributor in the development of hepatic insulin resistance, whereas FFAs from subcutaneous fat are dispersed in the peripheral circulation (300).

Macrophages are recruited into the adipose tissue by chemotactic gradient through various chemokines (MCP-1, LTBR) as the initial inflammatory stage. This initiates a feed-forward process, as ATMs in turn secrete their own chemokines, attracting additional macrophages (298).

Macrophage subpopulations are generally referred to as either *classically activated* macrophages (CAMs), also named M1, with a rather proinflammatory cytokine secreting signature, or the *alternatively activated* macrophages (AAMs), or M2, which secrete anti-inflammatory cytokines favouring tissue homeostasis such as IL-10 and IL-1 receptor antagonist (IL-1Ra).

The described ATMs polarization state does seem to inherently correlate with insulin sensitivity (301). However, this notion may be in part an oversimplification deriving from the *in vitro* setup in which bone marrow–derived cells were exposed to very exact growth factors, while their behaviours within the tissue are often much more dynamic and complex. Macrophages display phenotypic heterogeneity and high plasticity allowing them to change their polarization state in a wide range of more or less proinflammatory, in response to the local environment and intracellular signalling (302,303), with studies presenting contradictory results to the general paradigm. While it is not strictly within the scope and aims of this dissertation, the integration of metabolism with immunity, known as immunometabolism, is at the forefront of immunology research. It is in fact the cellular metabolism that shapes both immune cell state and fate, and contributes to infectious disease, inflammation, and cancer (304). In such a manner, polarized M1 macrophages activated by pro-inflammatory stimuli such as lipopolysaccharide (LPS) and IFN-γ, exhibit increased aerobic glycolysis through mTOR/HIF-1 signalling (305,306). In contrast, M2 macrophages are often polarized and enhanced by T-helper 2 (Th2) cytokines such as IL-4 and IL-13 exhibiting an increased mitochondrial metabolism through oxidative phosphorylation (OXPHOS)- and STAT6/PPARγ-mediated fatty acid oxidation (307–309). These findings underline the critical link between metabolism and immunity, as mitochondrial metabolism clearly

regulates macrophage cell function and in particular, the transition between aerobic glycolysis and OXPHOS plays a fundamental role in their polarization.

Regarding macrophage classification, their function and mediation of insulin resistance, studies based on glucose tolerance and insulin sensitivity analysis in lean and obese mice depleted of $CD206^{+}$ M2-like macrophages demonstrated an unexpected new role for a cluster of M2-like cells in adipose tissue. These M2-like ATMs increased the lipid burden of the existing fat cells, by creating a microenvironment that inhibited growth and differentiation of adipocyte progenitors - via the CD206/TGFβ signalling pathway - inducing hypertrophy rather than hyperplasia of adipose tissues, thus impairing insulin resistance (310). M1-like macrophages are overall increased in expression in obesity, secreting inflammatory cytokines resulting in insulin resistance. Obesity-induced recruitment of M1 macrophages into adipose tissue, and the resulting hypertrophy, is also directly responsible for its chronic inflammatory state (311). M1 macrophages induce a proinflammatory milieu within the adipose tissue by secreting tumor necrosis factor alpha (TNF-α). This cytokine acts directly on macrophage receptor TNFR1, with the immediate effect of caspase-8 and caspase-3 being cleaved and activated and inducing IκB phosphorylation to activate nuclear factor κB (NFκB). This in turn induces inflammatory cytokine production in the adipocytes resulting in lipolysis and hypertrophied adipocytes (312) as FFAs, especially saturated fatty acids (SFAs), are released locally within the tissue. This lipolytic inflammatory pathway further feeds into itself by activating the toll-like receptor TLR4 on macrophages further supporting the NF-κB pathway. And although the exact pathway for this mechanism remains still controversial (313), undoubtedly TLR4 does, although indirectly, regulate SFA-induced inflammation by altering macrophage lipid metabolism (314–316).

TNFα is also a central component in follicular formation, oocyte maturation, and androgen synthesis (317). By directly increasing the levels of MIP-3α in follicular fluid, TNFα plays a crucial role in the induction of follicular development and oocyte maturation that causes ovulation (318).

Proinflammatory interleukin-1β is propagated following inflammasome activation (319). NLRP3 (also known as NALP3) inflammasome has been reported to be activated by

cholesterol crystals present in macrophages (320,321), with a release of IL-1β cytokine that leads to a disruption in glucose metabolism and insulin sensitivity (322). The inflammasomes are among multimeric protein complexes structured into a sensor molecule NLRP3, the attached adaptor protein ASC, and a caspase. Formation of this intracellular multimeric protein complex is triggered by a range of substances that emerge during infections, tissue damage or metabolic imbalances (323). Assembly of the inflammasome in ATMs results in cleavage and activation of caspase-1, which proteolytically activates the pro-inflammatory cytokines (IL-1β) and IL-18. On a side note, inflammasome activation causes also a rapid, pro-inflammatory form of cell death called pyroptosis (324). Hence, high fat diet (HFD) - induced elevation of fatty acids, along with oxidative modifications caused by enzymatic attack of myeloperoxidase and lipoxygenases, or by reactive oxygen species leads to the release of bioactive lipids which are recognized by cholesterol receptors igniting the cascade (325). Based on these premises, a protective effect towards HFD-induced insulin resistance can be sought via deletion of NLRP3 or ASC or pharmacological inhibition of caspase-1 (326)

Abundance of circulating interleukin 6 (IL-6) and CRP, both sensitive physiological markers of subclinical systemic inflammation, positively correlate with obesity-induced insulin resistance (327). IL-6 is generally considered, produced in a variety of tissues, including activated leukocytes, adipocytes, and endothelial cells. C-reactive protein has hepatic origin mainly from IL-6-deriving biosynthesis, and is involved in the acute phase response. Both biomarkers have been considered as predictors of cardiovascular events (328–331).

Along with TNFα, both serum and follicular fluid of PCOS affected women commonly present increased levels of IL-6 (332) which may derive from activity of the follicular granulosa cells. In this regard, a meta-analysis of 25 case-control studies reported that this cytokine was significantly higher in women with PCOS independently of BMI as values were equally higher in lean and obese women alike. Interestingly, values were associated with the recorded IR and total testosterone levels (333). IL-6 does produce also contrasting anti-inflammatory effects (334,335) particularly in skeletal muscle (336) although the variations in its actions may strongly depend on the level of IR (333) and may also be highly tissue-specific between insulin target organs affecting the insulin sensitivity of each tissue differently. For CRP instead, prospective case control

studies in women age 45 and older, suggested a diagnostic potential as an independent predictor even after adjustment for obesity, clinical risk factors, and fasting insulin levels (327).

Other adipose-derived secreted factors or adipokines, that have pro-inflammatory activities in the context of metabolic disease are IL-18, C-X-C motif chemokine-5 (CXCL5), angiopoietin-related protein 2 and lipocalin 2 (337).

Clearly insulin resistance deriving from hypertrophic adipose tissue growth is driven by inextricably linked mechanisms based both on lipid accumulation in non-adipose tissue sites combined with a heightened sensitivity of the immune system, and overproduction of inflammatory cytokines from TLR4-activated infiltrating cells creating a dysregulated environment that can affect metabolic regulation in a variety of manners (338). In the last two decades, multiple studies have revealed the various inflammatory processes driven by adipokines and inflammatory cytokines, critically linked to metabolic pathways controlling the utilization of excess fat, which have a marked impact on insulin sensitivity (338–341). Among these, cytokines behave in a largely paracrine manner though exceeding levels can spill into circulation. This is why identifying tissue specific concentrations of both hormones and cytokines is highly relevant as they are evidently in different concentrations compared to the circulation which may not be representative of the endocrine effects on systemic inflammation.

1.3.4.2 Dendritic cells

Dendritic cells (DCs) are a heterogeneous group of mononuclear phagocytes, expressing high levels of major histocompatibility complex (MHC) class II (HLA-DR), which process antigens to present to T cells, serving as a bridge between the innate and adaptive immune responses. Their role is both essential for primary immune responses as much as maintaining immunological tolerance (342). Indeed, DCs consist of multiple subtypes with unique functions, which have been recently described according to a newly proposed taxonomy based on identified markers validating six DC subtypes corresponding in part to known DC subsets as well as to previously uncharacterized subsets. The main hystorical subsets, identifying CD11C+

conventional DCs (cDCs) and plasmacytoid DCs (pDCs) expressing CD123+, was reconsidered based on gene expression that reflected rather a spectrum of states which were enriched for either cDC-like or pDC like gene signatures. This led to the definition of a more encompassing DC subset, AS DCs (AXL+, SIGLEC6+), based on stronger T cell activation potential while pDCs confirmed their “natural interferon producing” role (IPCs)” and a milder T cell proliferation induction ability. cDC-like CD1C+ cells instead clustered separately into 2 distinct populations. Among these, CD1C_A identified by a major histocompatibility complex class II–like gene set and secreting higher levels of the immune mediators CCL19, IL-10, IL-12B, and IL-18. Conversely, CD1C_B expresses a CD14+ monocyte–like prominent gene set. Finally, a new CLEC9A+ DCs population, rising from the cDC progenitor.

Terminally differentiated or mature DCs can readily prime T cells by creating an enriched milieu of T cell stimulating inflammatory cytokines such as TNFα, IL-6, IL-11, IL-12, and IL-23, which, in turn, stimulate the outgrowth and activation of a variety of T cells (343). Early studies already proved this crosstalk not to be unidirectional only, from DCs to T cells, but is rather a “dialogue” as in fact CD40 and the TRANCE/RANK receptor on DCs are in turn ligated by the TNF family of proteins expressed on activated and memory T cells (344,345) stimulating DC survival and upregulation of CD80 and CD86 (346), secretion of IL-12(347,348) and release of chemokines such as IL-8, MIP-1α and β (346). However, DCs also play a key role in mitigating inflammation and regulating fat expansion in the visceral adipose tissue through PPARγ pathways (349). This happens as DCs activation of β-catenin prompts PI3K/Akt and subsequent IL-10 production, neutralizing IL-6 secretion (350).

DCs make up a significant fraction of hematopoietic cells in the follicular fluid (FF) (351) as part of the aseptic inflammation in ovulation. It is important to recognize that the cycling ovary, from follicular development, maturation, and ovulation and ultimately the formation of the corpus luteum till its regression, is a controlled tissue damage and healing (352) and is a tightly orchestrated inflammatory state (353). This microenvironment is mostly defined by the cellular components of FF (353). Proangiogenic and cytotoxic natural killer NK cell subpopulations ($CD56^{+}CD16^{-}$ and $CD56^{+}CD16^{+}$) which constitute a significant part of the bone marrow–derived cells present in follicular fluid, have been reported to account for 10% of the CD45+ bone

marrow–derived cell in the FF (354). However, DCs are even more abundant, making up ~15% of these cells (351), with a central role controlling key processes such as inflammation, angiogenesis, and tissue repair (355).

Follicular fluid in women with PCOS has been proved to lack equal amounts of DCs compared to their matched controls (356), which is highly relevant considering the discussed role of DC maturation and their action in overseeing inflammation. These findings also suggest a potential interruption in T cells recruitment and activation, another important aspect induced by follicular DCs, supporting the mechanisms leading to folliculogenesis arrest (357).

1.3.4.3 Innate Lymphoid Cells

Innate Lymphoid Cells (ILCs) develop from common lymphoid progenitor cells, featuring a lack of myeloid cell and dendritic cell phenotypical markers; described as innate counterpart of the T cell family (358). The prototypical ILC populations are NK cells and lymphoid tissue-inducer (LTi) cells, which are respectively, mediators of early immune responses against a diverse group of pathogens and tumor cells as well as responsible for the formation of lymph nodes during embryogenesis. They are a family of developmentally related cells involved in homeostasis by modulating immunity, tissue development and remodelling (358,359). As previously mentioned, during chronic systemic inflammation and metabolic dysregulation, a proinflammatory milieu is required for macrophages to switch from a homeostatic to an inflammatory state (360). The framework of macrophage activation does not seem to occur in an “on-off” bipolar mode, but rather transition across a spectrum of activation programs which may be partially determined by the influence of ILCs. Specifically, ILCs NK cells, may represent a crucial link between hypertrophic adipose stress and inflammation (361) as they regulate the activity of other immune cells, such as macrophages and DCs. Generally, in a regulatory loop manner, TLRs mediated recognition of pathogen by DC promotes NK cell proliferation, cytokine production, and cytolytic activity which then in return, shape DC population in order to boost a specific T cell activation towards a Th1- and CTL-mediated response (362,363). However, NK cells comprise a large number of distinct populations influenced by factors such as tissue localization and imprints by viral infections (364). For instance, VAT-resident NK cells can also recognize sterile

triggers of inflammation such as obesity-induced adipose stress via upregulated NCR1 ligands. In this setting, these unique NKp46+ (NCR1 in mice) *invariant* natural killer T cells (iNKT), in response to NCR1 signalling, release IFN-γ inducing M1 macrophage differentiation (361).

ILCs constitute a larger family that mirrors the activity of differentiated T cells. ILCs can be categorized as cytotoxic ILCs, represented by NK cells, and helper-like ILCs, with the subsets ILC1, ILC2 and ILC3 as innate counterparts of CD4+Thelper 1 (TH1), TH2, and TH17 cells (365,366) . ILC1 cells are defined by the production of the signature cytokine IFNγ and might best correspond to T helper type 1 (TH1) CD4+ T cells with the inability to produce TH2 cell- and TH17 cell-associated cytokines. However, although all ILC1 cells express T-bet, respond to IL-12 and IL-15 and share the ability to produce IFN-γ, only NK cells express EOMES (367). ILC2 cells, produce mainly IL-5 and IL-13 TH2 cell-associated cytokines in response to stimulation with the cytokines IL-25 and IL-33. Interestingly, in humans IL-4 and IL-5 producing ILC2 cells characterized by the transcription factor RORα+ and GATA3+ are decreased in obese individuals (368). Finally, ILC3 cells, are defined by their capacity to produce the cytokines IL-17A and/or IL-22 and engage in type 3 immunity, like TH17 helper T cells. They depend on the transcription factor RORγt for their development and function, and similar to the group 2 ILCs, these also depend on IL-7Rα. They represent a heterogeneous population which are prototypically known as lymphoid tissue-inducer cells and natural cytotoxicity receptor (NCR) positive or negative cells, NKp46- and NKp46+ ILC3s (368).

Circulating cNK cells are widely distributed throughout the body but various tissues have resident NK cells, termed tissue-resident NK (trNK) cells that are present in the liver (369), skin, kidney (370) and virgin uterus (371). These represent entirely different lineages of NK cells rather than different differentiation states, as trNK do not traffic freely remaining within the tissue (369,372,373) and are identified by differentially express receptors and transcription factors (374).

The uterus is one of the peripheral organs containing the highest frequency of NK cells. Indeed, NK cells are the most abundant among bone marrow–derived cells present in FF and their relative amount is correlated with the maturity of the ovarian follicle and

with the ovarian response to gonadotropin stimulation (354,375). These cells are tissue-specific, sharing phenotypic traits with both CD56bright and CD56dim NK cells (376). Commonly, the CD56bright NK cells are described as cytokine-producing cells with poor cytotoxic ability, whereas CD56dim NK cells are defined for their cytotoxic activity (377,378). In both mouse and human, uterine NK (uNK) cells are the most prominent immune cells that occupy the maternal-fetal interface engaging in complex interactions with the surrounding tissue, which impact their function (379). Several studies have reported that imbalances in uNK cell abundance are associated with infertility (380). This notion is in line with the earliest findings that essentially proved no cytotoxic ability towards prototypic NK cell-sensitive target cells from uNK cells (381). Pioneering work by Croy and colleagues brought this understanding one step forward by identifying a role for uNK cells in modulating placental vascular adaptation specifically through the release of IFNγ acting on endothelial cells and decidual stromal cells, in order to meet the nutritional demands of the growing fetus (382). Additionally, uNK cells have been reported to directly support embryo development by producing growth-promoting factors prior to the establishment of the placenta (383). Very recently, studies recognized novel unique subsets of human NK cells that only exist in repeated pregnancies (384), particularly during the first trimester (385) important in vascular modification of the placenta, proposing another previously unrecognized role in subsequent pregnancies to support placental vascular development. Whether they develop in situ from progenitor cells in the virgin uterus or home there from the periphery has been addressed using several approaches and remains of long-lasting interest (379). A two-wave hypothesis has been proposed, that is driven by uterine tissue remodelling events during pregnancy beginning with a local proliferation during decidualization process and minimal contribution from circulating NK cells, and a second wave that involves recruitment of cNK cells during the placentation process and vascular remodelling (374). The mucosal lining of the uterus is transformed from endometrium in the non-pregnant state to the decidua of pregnancy. This transformation is induced by increased progesterone and is associated with leukocyte infiltration (386). uNKs infiltrate the uterus at different anatomic sites such as the uterine mucosa and the decidual tissue, before and throughout pregnancy. Particularly in the first trimester, decidual NK cells (dNK cells) constitute the cellular majority,

>70%, among leukocytes in the placenta (387), together with some macrophages and a small number of T cells. B cells are virtually absent (388). They persist throughout pregnancy until term, which emphasizes the importance of uNKs not only initially, but also for maintaining pregnancy (389). The endometrium decidualization by the effect of progesterone which acts on stromal cells of the uterine lining throughout the second half of the menstrual cycle promotes homing and proliferation of peripheral blood NK cells (390). However, uNK cells do not express progesterone receptors and respond primarily to an increase in cytokines such as IL-15 within the uterine environment produced by mucosal stromal cells in the process of endometrium decidualization (391,392). This may be the case for the impaired recruitment of NK cells described in PCOS patients, deriving from the effects of both excessive circulating androgens and lower progesterone which reduce IL-15, as well as CXCL10, IL-18, and IL-12A (393) leading to the various downstream effects such as follicular dysplasia and ovulation disorder, and of course infertility (357). The reduced numbers of NK cells described among women with PCOS, particularly in the secretory phase of the cell cycle (393), are in line with our understanding of the role NK cells have in menstrual regulation and fertility (394,395).

1.3.4.4 T cells

T cells are a class of lymphocytes which can be divided into two main groups based on the incorporated receptor as CD4+ T cells and CD8+ T cells. A few studies have described an abnormal expansion of CD4+T cells both in ovary and peripheral blood among women with PCOS (396–398) although the exact subsets remain controversial at present. CD4+ T-αβ helper (Th) cell subsets can be subdivided into several different subsets based on their functionality, including Th17 cells, Th1 cells, Th2 cells, and regulatory T cells (Tregs), or characterized by their effector memory differentiation status.

Thymopoiesis starts with BM progenitor cells in the thymus, the primary site of T cell development where progenitors lacking CD4+ and CD8+ coreceptors undergo T cell receptor (TCR) rearrangement. Early studies based on peripheral T cells analysis, classified 3 main functional subsets deriving from thymic selection: naïve T cells, which have the capacity to respond to new antigens, memory T cells that derive from previous

antigen activation and maintain long-term immunity, and regulatory T (Treg) cells which keep immune responses in check (399).

Normally, thymocytes do not exit into circulation until they become mature T cells, acquiring single expression of CD8 or CD4 surface markers. However, thymics selection may already be susceptible to altered circulating steroids, particularly estrogen which has been proven to increase the gland's vascular permeability (400). This could cause early thymocyte discharge, bypassing the medulla, circumventing the final stages development, particularly of regulatory T cells (401). In this study, by utilizing an estrogen-induced anovulation and follicular cyst forming model of PCOS, a unique involvement of the thymus in cysts' genesis was proven. In fact, thymectomized, estrogen-injected mice had ovaries with corpora lutea. However, the condition was reversed by regulatory T cells infusion. This potential vulnerability for the human thymus to steroid action might be during the fetal stage with long-term effects on the ability to produce regulatory T cells which in female offspring may lead to PCOS.

Generally, androgens would appear to moderate the inflammatory capabilities of peripheral lymphoid cells(402), particularly T cells which are sensitive to androgens and express AR at all stages of development (403). Indeed, androgens have a significant influence on thymic size and the development of thymocytes through the intracellular AR within thymic epithelial cells. Males deficient in testosterone—whether due to castration or AR insensitivity—experience an increase in thymic size (404,405), which can be reversed by treatment with DHT. Beyond their effects on thymic structure, androgens enhance the elimination of self-reactive T cells by increasing autoimmune regulator (Aire) expression in medullary thymic epithelial cells (406), promote immune tolerance by elevating TGF-β production in the thymus (407) and limit the total number of T cells present in peripheral tissues. This may lead to the sex-based differences observed in T cell responses and cytokine production. T cells from non-autoimmune female mice generally respond more strongly to foreign antigens than cells from male mice (408), with male cells more often generating Th2 responses, while female cells tend toward Th1 responses(409).

Androgens may differently affect the development and activity of regulatory T cells (Treg) in males and females. Androgen supplementation in vivo has been shown to

expand regulatory T cell numbers in women with adrenal insufficiency and in female rats with experimental autoimmune orchitis. When bound to ligands, ARs directly enhance Foxp3 expression in T cells from rats and women during ovulation, but not in men, suggesting androgens can induce regulatory T cell development in women. Thymic development of regulatory T cells is also responsive to progesterone, and alterations of this pathway have been associated with gestational diabetes and transgenerational reprogramming of glucose homeostasis (410). A crucial step involves the thymic osteoclast differentiation receptor RANK which responds to increased progesterone promoting correct development of thymic Treg cells during pregnancy. Depletion of Rank in mouse thymic epithelium showed an impaired accumulation of Treg cells both in the placenta and in visceral adipose tissue, resulting in an increased number of miscarriages, tissue inflammation, enhanced maternal glucose intolerance, fetal macrosomia, and a long-lasting transgenerational alteration in glucose homeostasis. All features which were restored via transplantation of normally developed Treg cells.

Hence, these $CD4^+CD25^+$ T regulatory cells represent a crucial subpopulation, balancing immune homeostasis and autoimmunity. Low Tregs have been correlated with spontaneous recurrent abortion (411) and pre-eclampsia (412). There are two main subsets: naturally occurring regulatory T cells (nTregs), originating from the thymus gland, and induced regulatory T cells (iTregs) that instead derive from the peripheral lymphoid tissues. Treg pool of normal fertile women is shown to expand throughout the follicular phase of menstrual cycle, concurrently with estrogen (413), essential to facilitate tolerance for a successful implantation. Women affected with PCOS have significantly lower numbers of circulating peripheral $CD4^+CD25^+CD127^{-/low}$ Tregs during the early follicular phase compared with healthy controls (414). The relevance of these frequency values at this particular menstrual stage is because it defines a state of baseline, independent of any effects from ovarian steroid hormones which are commonly lowest at this point. Treg expansion during follicular phase was shown to be defective in women with PCOS (414). Although estrogen may directly affect Treg cells development via thymus permeability, this might not be the case in PCOS woman as their levels of circulating E2 commonly fall within the normal range. One alternative hypothesis may be an inherent disability to induce

peripheral Tregs (414). A notion that is further strengthened by the significant correlation between LH variations and Treg percentages. Indeed, the LH-induced dual activation pathway of JAK2, STAT5B and PI3K/AKT proved in rat ovaries (415), are all key steps in Treg commitment.

T cells have been described in nearly every organ and tissue of the body, with different immune responses associated with relative anatomic compartments and different life stages. Compartmentalized functions and properties of tissue-resident memory T cell (Trm) subsets suggest an anatomic complexity and tissue-based immunity for the T cell response associated with disease processes. Functional studies based on core signatures proposed a dual role for human Trm cells, both protective and regulatory. Human Trm cells produce cytokines associated with protective immunity such as IFN-γ, IL-17, TNF-α, and IL-2, but also the immunoregulatory cytokine IL-10 (416–418). Moreover, human Trm cells express surface receptors known to potently inhibit T cell function including PD-1, LAG3, CTLA-4 (416,417,419), and CD101 (417), a molecule associated with inhibition of proliferation.

A role for T reg cells in adipose tissue derived chronic inflammation and insulin resistance has been mechanistically proven by preclinical murine studies. Adoptive transfer of Tregs to diet-induced obese mice, showed an immediate improvement of insulin action (420). This is in line with the finding that obesity causes a drastic decrease of overall T regulatory cells resident in VAT, to approx. 20% of adipose resident T cells (ARTs) (421). This drop in AT Tregs is accompanied by a greater abundance of pro-inflammatory interferon gamma (IFNγ)- secreting CD4+ helper cells (Th1) and CD8+ T cells (422,423). Although limited studies have investigated T cell flow in adipose tissue in humans, a similar process may be occurring, as adipocyte MHCII upregulation occurs in obesity (421) and expression of adipose tissue Th1 markers and IFNy also increases, while expression of FOXP3 decreases (420,424).

When observing the differentiation of activated CD4+ T cells, PCOS inflammation may reflect a mixed type 1 and type 3 immune response phenotype. Type 1 responses are shaped by the production of IL-12 by DCs and macrophages driving the expression of the transcriptional regulator T-bet (425). This generally leads to the activation of natural killer (NK) cells and group 1 innate lymphoid cells (ILC1s) (365), followed by the

activation of T_H1 cells and cytotoxic CD8+ T cells. Findings in follicular fluid from PCOS women reported lower values of IL-13, while IL-12 seems to be commonly increased (426). The resulting increase of lymphoid interferon-γ (IFNγ) production enhances macrophage activation and cytotoxic molecules, such as perforin and oxygen radicals. radicals. Type 3 responses are supported by DCs and macrophage release of IL-1β and IL-23(427). Preclinical studies showed activation of key transcription factor STAT3 and the expression of RORα and RORγ (428) leading to higher induced circulating ILC3s and serum T_H17 cells often described in PCOS (429). The resulting cytokine environment is strongly IL-17 and IL-22 based. Indeed, while type 1 and type 3 responses are well-documented in autoimmune inflammatory diseases, in PCOS the driving inflammatory factors may be more subtle and cumulative, rather than overt and will be explored further in a following chapter on PCOS and autoimmunity. Increasing evidence has been pointing out to a strong gut microbiota dysbiosis in PCOS and loss in the diversity of bacterial symbionts (430), and have been a central focus of our group's research for several years (428,431). Combined with increased intestinal permeability, this may potentially be a source of chronic inflammation from gram negative bacteria derived LPS traversing the “leaky gut” wall into the circulation (432). The resulting type 1 interferons while fending against bacterial metabolites may result in organ-specific inflammation (433). Coincidentally, the inflammation in metabolically active tissues in PCOS does lead to enhanced type 3 responses (434), higher blood glucose levels and compromised cardiovascular health, all while type 2 responses are inhibited. The accumulation of Th1 and Th17 cells both in circulation and in tissues of interest, ovary and VAT, leads to systemic proinflammatory environment and heightened immune sensitivity.

1.3.4.5 B cells

B cells are well recognized for their relevance in various chronic inflammatory and autoimmune diseases (435,436) and more recently also for their role in obesity-related insulin resistance (437). B cells can also migrate to inflamed VAT tissue, regulating both systemic and local tissue inflammation (438,439), and may undergo functional and phenotypic changes during insulin resistance (438,440). However, the relationship between B cells and PCOS is relatively novel, perhaps also because of the general

lack of these specific immune cells in the female genital tract (441). Recent studies on peripheral B cells have shown that women with PCOS exhibited increases in the frequencies and functional activity of CD19+ B cells (442). More specifically, this study reported an expanded proportion of Naive B cells, an important source of precursors for pathologic autoantibody-secreting cells (443). However, percentages and numbers of memory B cells (CD19+CD27+CD38dim), plasmablasts (CD19+CD27+CD38hi), and regulatory B cells (CD19+CD24hiCD38hi) were not significantly different between control subjects and women with PCOS. Yet, levels of circulating TNFSF13B or better known as cytokine B cell activating factor (BAFF), a supporting cytokine of B cell survival (444), were distinctly higher in women affected with PCOS. It is yet unclear in what manner might the hyperandrogenic hormonal milieu in PCOS affect B cell populations and subsets in their inherent functionality.

From a developmental perspective, B cells are under intense selective pressure as they need to mount a vast repertoire for antigen-specific immune responses based on a highly diverse set of B cell receptors (BCRs) against both pathogen-derived antigens as well as potential neoantigens. This occurs via a random recombination of exons, a process which is largely driven by BCR signalling. The process called V(D)J recombination inherently produces a large number of self-reactive B cell clones (445), which therefore requires tightly controlled mechanisms of immune tolerance. In the bone marrow, after rearrangement of immunoglobulin heavy-chain variable region genes, progenitor B cells are first selected by central tolerance for expression of a functional pre-BCR. They then undergo affinity maturation in germinal centres (GCs), where they are once more selected based on BCR affinity to antigen (446). V(D)J recombination, class-switch recombination (CSR), and hypermutation-driven affinity maturation are critical for the development of a wide spectrum of pathogen-recognizing B cells. However, due to the high frequency of DNA double-strand breaks, B cells are at an ~300-fold increased risk of malignant transformation (447) and additional tolerance checkpoints are fundamentally necessary.

The expression of the AR at various stages of development provides some indication of the level at which androgens influence immunity. B cells are primarily sensitive during development, as bone marrow stromal cells and B cell precursors, but not

peripheral B cells, express AR (402). Several studies have found strong correlations between low testosterone and elevated numbers of B cells (448–450). Already from gestation, Lundell et al. demonstrated how males and females are subject to differential genetic- and/or hormonal-driven regulation of B cell lymphopoiesis, based on the positive correlation between DHT levels at birth and the frequency of immature B cells (451). Testosterone suppresses B frequencies both in the bone marrow and spleen, via pathways that can be, either directly activating the AR (452) or, respectively, indirectly limiting B cell expansion (444). Specifically, splenic B cell regulation in mice involves a testosterone-mediated signalling to the sympathetic nervous transmission and variations of splenic noradrenaline levels. In this manner, testosterone indirectly promotes increased splenic noradrenaline levels, limiting expansion of B-cell activating factor (BAFF) producing fibroblastic reticular cells (FRCs) and consequently leading to depressed splenic B cell numbers and circulating BAFF levels. This positive correlation between testosterone and circulating BAFF may not apparently explain what has been shown in PCOS (442), yet it does suggest inherent potential indirect mechanisms of B cell activation which remain elusive and may not rely exclusively on AR action. One candidate mechanism might be the excessive BAFF levels, as they do mention that serum BAFF was higher in female compared to male mice, which is also in accordance with a previous report of sex differences in BAFF levels among wild-type controls (453).

Extensive studies of the peripheral blood in mice, the peritoneal cavity, spleen, and other lymphoid tissues have identified two broad classes of B cell subsets, B1 and B2, based on their developmental origin. B2 cells, which represent the principal subset, originate from the bone marrow and include follicular as well as marginal zone B cells. B-1 cells are instead characterized as long-lived, self-renewing cells and have been described as two distinct subsets, B-1a cells, also described as CD5+ B1 memory cells, the major producers of “natural” polyreactive IgM, and B-1b cells which support humoral responses towards T cell-independent antigens (454–456). These so called natural pre-existing antibodies (NAbs) arise spontaneously early in life without apparent antigen exposure (457,458). Based on these premises, a human B1 cell equivalent has been proposed identifying a population present in umbilical cord and adult peripheral blood with similar spontaneous IgM secretion capabilities, that express

the unique phenotype of CD20+CD27+CD43+CD70− (459). These natural polyreactive IgM antibodies deriving from B1 cells have been shown to cross-react with oxidized forms of serum lipids (i.e. oxLDL) (460). Recent findings support a mechanistic role for IgM antibodies in providing homeostatic housekeeping functions in increased oxidative stress conditions by preventing the excessive accumulation of cellular debris and modified LDL particles (461). Furthermore, B-1a cells act as principal anti-inflammatory cytokine IL-10 producers (462).

More than one study has pointed out that in HFD conditions and IR state, B2 cells display a rather pathogenic behaviour (438,439). For example, when submitted to a HFD, mice lacking B cells (B^{null}/muMt-) have a more favourable glucose metabolism and reduced IR compared to controls (438). The reconstitution with B2 cells however, extracted from diet-induced obese (DIO) mice and placed into obese muMt- mice immediately affected all metabolic values, suggesting these cells to be potential drivers of inflammatory insulin resistance. An important detail, when transferring isolated effector elements of B cell function such as IgG from similarly obese mice to B cell lacking recipients, variations of inflammatory parameters only occurred when muMt- mice had already been previously subjected to a similar high fat diet. Interestingly, for these B cells to react to a prior conditioning, exposing target autoantigens, was necessary in order to produce proinflammatory and adverse metabolic reactions, which may be the case in the specific hyperandrogenic hormonal milieu in PCOS.

B cells promote metabolic inflammation and IR through multiple pathways which include release of proinflammatory antibodies and cytokines which regulate T cell activation. B2 cells can react to T cell dependent antigens (463) all while also orchestrating a more Th1 engaged activity through release of IFNγ, IL-12, and TNFα or favouring a more Th2 based response via IL-2, IL-4, and IL-13 (464). Among the central mediators of T cell polarization are the regulatory B cells (“Breg” cells) (465). During obesity induced tissue inflammation, B cells infiltrate VAT and aggregate around stressed adipocytes forming “crownlike structures” (CLSs) (298,438) with the purpose of identifying potential antigens and regulating both T cell and macrophage. MHC-dependent inducing of T cell IFNγ expression is a key aspect in B-T cell modulation. This interaction is reciprocal as T cells in return can generate feedback through CD40 – CD40L interactivity which modulate which cytokines B cells should

continue producing, as well as B cell development and class switching (466). These T cell cues are crucial in directing inflammatory reactions leading to IR, as noted in mice lacking both T and B cells (RAG KO) which upon receiving B cells from wild type mice exposed to high fat diet, exhibited quite mild variations in glucose metabolism (438), suggesting that the action of B cells towards insulin resistance might be tightly dependent on other cells in order to maintain activation of inflammatory metabolic parameters.

Another inflammatory characteristic of the visceral adipose tissue is the production of BAFF, which has been proven to increase proportionally with lipid accumulation and hypertrophy, associated insulin resistance (467). Hamada et al. isolated this BAFF secretion in vitro by activating the NF-κB pathway through oxidative stress. This direct interaction with VAT adipocytes-deriving cytokines can modulate B cell, as excess availability of BAFF can act on BCR-engaged autoreactive transitional B cell clones and promote their maturation into follicular (Fo) and marginal zone (MZ) B cells, enhancing the possibility for autoimmunity (468,469).

From a metabolic standpoint, B cells are by default in a state of chronic energy depletion (470). This is important as the rapid transitions between the quiescent and activated state are associated with dynamic reprogramming of cell metabolism and variations in signal transduction crosstalk, including the signalling roles mediated by metabolites and nutrients (471). Low ATP reserves and restricted mitochondrial number and volume are critical in controlling both normal B cell development and avoiding an unrestricted growth of pathogenic cells that may be generated during the process. Indeed, compared with cell types that have high levels of energy metabolism, such as skeletal and cardiac muscle cells (containing more than 2,000 mitochondria per cell), mitochondria number and mass are generally lower in hematopoietic lineages and even lower in B cells (3–8 mitochondria per cell) (472). Jang et al. (473) demonstrated that mitochondrial function is crucial in directing the fate of B cells upon activation by regulating levels of heme- and mitochondria-derived reactive oxygen species (ROS). Interestingly, mitochondria are also the principal subcellular target of metformin, which action was very recently proven to directly target the mitochondrial respiratory chain activity (474) which among other things, diminishes B cell's output of the cytokine TNF-α (475). Xiao et al. went on to prove that metformin has a profound

impact on mitochondrial morphology and membrane potential in B memory cells, which clearly impacts the respiratory chain activity and the production of matrix metalloproteinases (MMP) and ROS. Metformin also reprograms B cell expression of its glucose transporters Glut 1 and Glut 4, and the transcription factors HIF1α and c-Myc. This study proved the mTOR pathway to be the cellular target of metformin. Xiao et al. utilized rapamycin to trace back the mTOR phosphorylation activity (476,477), to the regulation of TNF-α expression in B cells in women affected with PCOS.

The process for B cell immune reactivity is based on a mechanism defined as class switching. Obesity is an independent factor responsible for a process very much similar to inflammaging leading to functional impairment of immune cells. Increased serum TNF-α in obesity is associated with distinct B cell defects that are critical for optimal humoral immune responses (478). The pioneering work by Frasca D. et al. showed that, similar to aging, obesity affects both frequencies and functionality of B cells, often degenerating to the secretion of autoimmune IgG antibodies and expansion of unique memory B cell clusters. Already a first publication in 2011 (438), by applying a protein array for more than 9000 selected “self” antigens, first proved that insulin resistant individuals present higher frequencies of circulating IgG autoantibodies. Frasca et al. went one step further by demonstrating how several mechanisms of adipose tissue meta-inflammation, or low-grade systemic inflammation, such as hypoxia, cell cytotoxicity, DNA damage are responsible for the release of “self” antigens, which stimulate class switch and the production of autoimmune IgG antibodies (479). They explored a potential expression of the transcription factor T-bet in B cells, along with surface marker CD11c (479). Encoded by the tbx21 gene, T-bet is known to be associated with the secretion of IgG2a/c antibodies in mice (IgG1 in humans) (480–482), which are pathogenic (autoimmune) antibodies. Also, T-bet expressing B cells commonly express CD11c (Itgax) surface marker, an integrin involved in autoantigen presentation (483). T-bet+CD11c+ B cells are associated with immunosenescence and are expanded in healthy elderly individuals (484) but have also been reported in patients with autoimmunity, promoting for example the secretion of anti-chromatin IgG in SLE patients (485). These findings describe another underlying process in human adipose tissue which contributes to establish and maintain local and systemic inflammation. Staining for germinal centres (GC) identified both B (CD19+CD10+IgD)

and T (CD3+CD4+PD1+CXCR5+) GC in the SAT (479), with B cells being mostly in the Dark Zone. Germinal centres are specialized microenvironments that generally develop in secondary lymphoid organs, such as the spleen and lymph nodes, to facilitate long-lived T-dependent humoral immunity by generating antibody-secreting plasma cells and memory B cells. GC are formed when activated B cells from antigen encounter, upon receiving survival and co-stimulatory signals by CD4+ T cells, seed the GC response. (486) These GC B cell precursors begin to rapidly divide, creating a distinct compartment defined as the dark zone (DZ) , in opposition to the Light Zone where T follicular helper and T follicular regulatory cells await as gate keepers for positive selection. It is in the DZ of the GC that B cells rapidly proliferate, undergo clonal expansion, class-switch recombination (CSR), somatic hypermutation (SHM) of VH genes and affinity maturation (487). However, the formation of GC within the SAT may generate early extrafollicular plasmablasts that secrete low affinity antibodies (488), capable of exacerbating obesity-associated conditions enhancing local inflammation by activating complement and Fc receptors on immune cells, while also exerting detrimental effects systemically by targeting distinct clusters of self-proteins.

The growing attention for extra follicular somatic B cell hypermutation and affinity maturation in peripheral naïve B cells has led to an increased awareness for distinct differentiation fates upon activated naive B cells (aNAV). In this regard, an important body of work developed between 2007 and 2020 led by Jenks and Ignacio Sanz in recognizing the significance of exhausted atypical memory cells in Systemic Lupus Erythematosus allowed for a comprehensive interrogation also in other fields of immunology and inflammation research on the effects of chronic inflammation on peripheral B cell alterations. In 2018 they published a thorough description of a distinct subset of CXCR- CD11c+ aNAV-deriving B cells which seemed to endow various chronic autoimmune and inflammatory diseases with properties resembling antibody secreting cells (ASC) which were directly associated with disease severity (489). These findings subdivided age-associated class-switched B cells lacking IgD and the B cell memory marker CD27 (double negative B cells; DN) into two major fractions: DN1 cells, the dominant fraction in healthy individuals, with a CXCR5+, CD21+ phenotype; and the DN2 cells, with a CXCR5-, CD21- phenotype, representing pre-plasma cells (PC) of pathogenic relevance. The DN1 B cell subtype constitutes the

majority of the DN B cell group found in healthy individuals (490). CD21 acts as a coreceptor that, alongside CD19, enhances signalling through the B-cell receptor (BCR) and aids in antigen presentation to T cells (491). CXCR5, in turn, is the receptor for chemokine CXCL13, which directs B-cell migration into germinal centres within lymphoid organ follicles, facilitating the follicular B-cell response (492). Evidence so far indicates that DN1 B cells may serve as precursors to switched memory B cells. For example, Jenks et al. (2018) noted gene expression patterns in DN1 B cells that closely resemble those of switched memory B cells, while Stewart et al. (2021), using pseudotime analysis, proposed DN1 B cells as potential predecessors to IgD−CD27+ memory B cells. Given these findings, it's plausible that DN1 B cells could also be involved in the follicular pathway.

DN2 B cells have been highly characterised. This subset of DN cells also seemed to be characterized by higher expression of CD11c, Toll-like receptor-7 (TLR7) surface markers and a T-bet transcriptional network. In an attempt to arrange these unique activated B cells, Jenks et al. (2018) define this cluster as sharing both phenotypic and functional features and similar transcriptomes with aNAV, as well as clonal sharing with circulating plasmablasts (PB). Furthermore, proving an enhanced responsiveness to TLR7 in an interleukin-21 (IL-21)-mediated fashion these highly reactive extrafollicular derived PC precursors underscore the prominent participation of a distinct extrafollicular differentiation pathway in autoimmunity. This may be relevant to PCOS, which represents a state of elevated immune sensitivity to cytokines and chemokines and potentially a context of persistent antigenic stimulation and chronic inflammation which might affect the adaptive immune cell repertoire in a similar fashion.

1.4 PCOS treatment management

Effective management of PCOS as a chronic condition with heterogeneous clinical phenotypes and long-term health risks, requires a multidisciplinary approach tailored to the patient's symptoms and health goals. This chapter will briefly outline key components of PCOS management, including lifestyle modifications, pharmacologic interventions, and targeted therapies.

Lifestyle changes are foundational to managing PCOS, especially in order to address potential aggravating obesity and to reduce the state of insulin resistance (493). This analysis of randomised controlled trials introducing behavioural interventions and lifestyle changes such as a balanced diet and regular exercise found improved insulin levels, supported weight loss, and enhanced hormonal balance, which may alleviate PCOS symptoms. Indeed, studies indicate that already a modest weight loss of 5-10% can improve menstrual regularity, ovulation, and insulin sensitivity (494). Hence, a diet that helps regulate blood sugar levels may be crucial for women with insulin resistance, and regular physical activity can improve insulin and leptin sensitivity, and ovulation (495). Additionally, behavioural therapies, such as cognitive-behavioural therapy (CBT), have shown relative efficacy in promoting adherence to lifestyle changes and managing anxiety and depression, which are commonly associated with PCOS (496).

Pharmacologic Interventions are often prescribed to address the hormonal imbalances and the individual specific symptoms associated with PCOS. Combined oral contraceptives, containing estrogen and progestin, are frequently used to regulate menstrual cycles, reduce androgen levels, and alleviate symptoms like acne and hirsutism (497). Anti-androgens such as spironolactone may be used to reduce androgen levels, thereby addressing symptoms like hirsutism and acne although they can interfere with fetal development, so are typically considered alongside the use of contraceptives when the intent is also to prevent pregnancy (498). Given the high prevalence of insulin resistance in women with PCOS, insulin sensitizers like metformin are often prescribed to improve insulin sensitivity and reduce hyperglyemia (499). Interestingly, the anti-inflammatory action of metformin has been widely adopted for its antitumor, antiaging, and neuroprotective effect in various other conditions (500,501). Recent findings previously discussed, have proposed that metformin alleviates PCOS by diminishing the release of TNF-α from pro-inflammatory B cells by inducing mitochondrial remodelling (475). For women with PCOS who wish to conceive, ovulation induction agents such as clomiphene citrate or letrozole are used to induce ovulation (502) In this study, Letrozole was been shown to improve ovulation rates and pregnancy outcomes, often serving as a preferred first-line option for ovulation induction in PCOS.

Finally, managing specific symptoms like hirsutism, acne, and infertility may often require targeted therapies. While costly, these options can significantly improve quality of life by addressing visible symptoms or assisted reproductive technologies (ART). Indeed, possibly the most overlooked aspect is how PCOS often impacts mental health, as body image issues, weight gain, and infertility can lead to anxiety and depression. Mental health support has been proven to be crucial in PCOS management as PCOS is associated with elevated rates of anxiety and depression (496).

Managing PCOS requires a comprehensive approach that includes lifestyle modifications, pharmacologic treatments, targeted therapies, and mental health support as well as long-term monitoring and follow-up. Addressing both the physical and emotional aspects of the condition can improve symptoms, enhance quality of life, and reduce long-term health risks. With ongoing monitoring and personalized care, women with PCOS can effectively manage the disorder and achieve better health outcomes.

1.5 PCOS and autoimmunity

Sex steroid hormones profoundly affect the immune system, influencing health and disease via regulation of immune cell activities while also modulating target-organ susceptibility to immune-mediated damage. Insight into these effects has provided important clues to the sexual dimorphism of immune-dependent disorders. Various systemic autoimmune disorders are commonly more prevalent in women (503,504), while cancer seems to demonstrate an opposite pattern, suggesting that testosterone, the main androgen, may protect against autoimmune disease (505). Intriguingly, patients of either sex with autoimmune disorders also demonstrate lower serum concentrations of androgens resulting from various causes for example hypopituitarism or Klinefelter´s syndrome in men (506–508). Another quite evident report is the risk joint appearance of SLE which increases up to 18-fold in Klinefelter patients proving clinical remission following testosterone substitution (509).

Consistent with these findings we know that castration has proven to aggravate disease in different animal models of experimental autoimmune disease (510,511) while treatment with both testosterone and the endogenous AR agonist dihydrotestosterone seems to ameliorate conditions both in experimental lupus and arthritis, supporting a general notion of a protective role of AR activation. In this context, the significance of BAFF regulation as a mediator of a beneficial testosterone/AR agonist in experimental disease models remains unclear. Conversely, estrogens excess has been linked to different autoimmune diseases by studies on women of reproductive age (512), as well as studies in hypogonadal men having relative excess of estrogens. Expression of serum BAFF levels tend to be higher in female compared to male mice (444). Interestingly, both hormones seem to suppress bone marrow B lymphopoiesis (513,514) while differing in their effects on splenic B cell number and BAFF. This led to an overall assertion that estradiol accelerates autoimmunity (503) and elevates BAFF production, whereas testosterone has the opposite effects (452).

Nonetheless, a crucial bulk of studies addressing sexual dimorphism of immune-dependent disorders revolve around testosterone deficiency studies induced by castration on male mouse models of autoimmune disease, while the effect of androgens on susceptibility and severity in women is generally less well understood. At periovulatory to pregnancy levels (515), estrogens, and in particular 17-β estradiol (E2) has mainly anti-inflammatory effects, by inhibiting production and signalling of pro-inflammatory cytokines, such as TNF, interleukin (IL)-1β and IL-6, and natural killer (NK) cell activation, rather inducing expression of anti-inflammatory cytokines which favour a T helper 2 (Th2) phenotype (516), such as IL-4, IL-10 and transforming growth factor (TGF)-β, and by activating regulatory T cells (Treg) (517). However, E2 as well as prolactin act as enhancers of humoral immunity. At lower concentrations, E2 stimulates TNF, interferon (IFN)-γ, IL-1β and NK cells, while it enhances antibody production by B cells both at high and low concentrations (516). Similarly, prolactin promotes the development of CD4+ T cells, increases both antibody and pro-inflammatory cytokine production (518). In line with this notion, changes in the severity of autoimmune diseases have been observed during pregnancy, coinciding with the highest levels of circulating estrogens and progesterone (519). In order to induce maternofetal immune tolerance, essential to maintaining pregnancy, one of the crucial

adaptations is the shift at implantation from a pro-inflammatory Th1/Th17 response, which promotes rejection, toward a Th2/Treg cell response that promotes tolerance and inhibits NK cell cytotoxicity (519). Possibly due to these adaptive changes in immune system function, some autoimmune diseases may behave differently during pregnancy. For instance, pregnancy is associated with an increase in disease flares in SLE, this effect being related to the increased Th2 response and enhanced production of pathogenic autoantibodies (520). Conversely, pregnancy has a protective effect in Th1-dominant immune diseases, like RA and MS.

While one might imply that higher androgen levels in women affected with PCOS hinder against degenerative autoimmune diseases, very often these women experience quite the opposite with recurring joint appearance of type 2 diabetes mellitus (15), different forms of thyroid disorders, particularly Hashimoto's thyroiditis (HT) (2). Circulating anti-histone and anti–double-stranded DNA antibodies are also more present among PCOS affected women (521). Nonetheless, a mechanism based on autoimmunity to explain the pathophysiology of PCOS remains yet to be defined (16–18). Increasing literature has described the current search for novel informative parameters to help identify PCOS earlier in its development, support treatment and stratify the patients. These include molecular analyses testing for associated gene variants and genetic alterations, metabolome and proteome analyses from different matrices, immunological approaches to identify alterations in cells of the immune system and circulating autoantibodies, or microbiome analyses (159,522–525).

One hypothesis has considered the possibility that the irregular estrogen values may be opposing any beneficial effects on the immune system deriving from AR activation (18). Use of the androgen receptor antagonist, flutamide acts indirectly also on the estradiol and progesterone be regularizing their endocrine feedback (526) preventing androgen induced decrease in insulin sensitivity, and reversing DHT-induced hepatocyte insulin resistance. Generally, progesterone acts as an intermediary, suppressing estrogen induced immune stimulation; a feedback system that might be impeded in PCOS as lower level of progesterone may be leading to over-stimulation of the immune system (18).

As joint appearance of both hypothyroidism and PCOS has a prevalence of 70% within families and twins, some studies have traced common polymorphisms within related genes such as fibrillin 3 (FBN3), gonadotropin-releasing hormone receptor (GnRHR) and CYP1B1 encoded to act on estradiol hydroxylation (527). Fibrillins act synergistically with transforming growth factor-beta (TGF-β) which stimulates regulatory T cells (Tregs) mitigating excessive immune responses. TGF-β1 is generally lower in both HT and PCOS, although the added presence of an identified allele in the FBN3 gene (D19S884) resulted also in lower circulating frequencies of Tregs and vitamin D deficiency(2).

The cumulative effect resulting from lower levels of both TGF-β and Tregs may coincide with a higher susceptibility due to the lack of inhibitory processes. On a side note, the role of vitamin D in immune regulation may deserve further investigation. In a study of 206 PCOS women, 72.8% were found to be with 25(OH) vitamin D insufficiency (<30 ng/mL) which was associated with significantly worse metabolic characteristics (528). However, this study presented numerous limitations and might rather refer to reversed causality, and more comprehensive intervention trials to evaluate the effect of vitamin D supplementation on metabolic disturbances in PCOS women are needed. Obesity is an independent risk factor for lower levels of vitamin D (529). This might be of relevance considering how common among patients with PCOS are both metabolic syndrome and obesity. In a study by Orbach et al. (530) 1029 patients with various autoimmune disorders such as scleroderma, polymyositis and dermatomyositis, antiphospholipid syndrome, rheumatoid arthritis and SLE presented lower levels (average of 9.3–13.7 ng/mL) of 25(OH)D than controls.

It is well-established that the gut microbiota affects the innate and adaptive immune responses, as well as being influenced in its composition by the same immune system in a bidirectional manner. This interaction can have important consequences for the development of inflammatory diseases (531). Not surprisingly, a role for gut microbiota in the sex bias in autoimmunity has been recognized by different preclinical studies and the existence of specific gut microbiota patterns that appear to be associated with autoimmunity. For instance, in the spontaneous NOD model of type 1 diabetes which is characterized by a strong female bias, disease rate was notably similar in germ-free female and male NOD mice, possibly suggesting a male-specific microbiota

composition may play a protective role (532). In the same study, a mechanistic approach was demonstrated, at least partially, through gavage of male NOD-derived intestinal microbiota by female NOD weanlings which induced elevated testosterone levels and increased protection from the developing type 1 diabetes when compared with unmanipulated females. This may suggest a microbiota-mediated alteration of the immune profile, potentially affecting metabolism of sex hormones which is supported by with sex-specific differences found in microbiota composition after puberty, underlying the interaction between sex hormones pathways and gut macrobiotic metabolites (532,533). Previous research also demonstrated an opposite effect, such as beneficial anti-inflammatory influence deriving from treatment of lupus nephritis with *Lactobacillus* that were uniquely observed in female and castrated male mice but not in intact males (534). These findings were further confirmed in mouse models of lupus and RA which demonstrated an equally significant difference between male and female adult mice gut microbiota composition (535,536). However, the role of gut microbes in autoimmunity and particularly their interaction with hormones remains mostly within the sphere of preclinical research and is still poorly characterized in humans although novel strategies carefully observing probiotic supplementation have shown to beneficially influence metabolic and inflammatory biomarkers in autoimmune affected patients (537).

A newly described autoantibody capable of binding to G protein–coupled receptors (GPCRs) has widened our understanding of how GPCR signalling pathways may affect pathogenesis of various diseases (538–540). Interestingly, while natural GPCR ligand binding desensitizes its natural receptor, conversely GPCR-directed autoantibodies do not seem to induce such desensitization (541). Furthermore, by allosterically binding to the non–pocket-related receptor structures of the GPCR receptors, they additionally alter any further natural ligand interaction (539,542). This is particularly relevant within the specific pathogenesis of PCOS, as GPCRs are highly present on ovarian cells and play crucial roles in regulating ovarian functions, such as follicle development, ovulation, hormone synthesis, and secretion. Among these are Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) which have already been described but also various prostaglandins attach to GPCRs, neuropeptides and neurotransmitters such as Kisspeptin and Serotonin which play a role in ovarian follicle growth,

steroidogenesis, and oocyte maturation. As previously described, the hypothalamus-pituitary-ovary (HPO) axis controls ovarian development and function, from follicle recruitment to oocyte selection, as well as regular cycling and steroid hormone biosynthesis (543). This is governed by the rhythmic release of hypothalamic GnRH into the blood system which coordinates the synthesis and release of the pituitary gonadotropins. In this process, the GnRH-receptor on gonadotropic cells plays a key role in translating circulating GnRH to FSH and LH. Target organs, such as the ovaries, are greatly influenced by the relative amounts of the circulating gonadotropins, the so-called LH/FSH ratio, which is elevated in PCOS (544). Endocrine gonadotropin specific GPCR receptors are established targets in autoimmune diseases. For example, the receptor for thyrotropin (TSHR) in autoimmune thyroid diseases, particularly Graves' disease (545). Given these circumstances, it is conceivable to assume the possibility of autoantibodies targeting the GnRHR in the pathophysiology of PCOS as well as the FSH receptor and the LH receptor representing disease-relevant targets in human subjects, as the TSHR does in Graves' disease (522,546).

In the first case, this hypothesis was considered by Kem et al., having already described multiple syndromes driven by GPCR-Aab activation inducing autoimmune reaction (547–549). By using a cell-based calcium flux GnRHR bioassay, they found a proportional correlation between the concentration of PCOS-deriving IgG and its potential to stimulate the second extracellular loop (ECL2) of GnRHR.

In the same year, Hongliang Li published a follow up of the same project (550) demonstrating how immunization of a female rat model (IMM) with GnRHR ECL2 did in fact induce a PCOS-like metabolic phenotype, with both hyperandrogenism and insulin resistance. Interestingly the immunized group developed these features independently of increase in body weight or fasting blood glucose levels. Therefore, when performing a more in depth analysis in the IRS/PI3K/Akt signalling pathway components in liver and muscle they found the IMM group to have a significant increase in insulin-stimulated serine phosphorylation of IRS-1 paired with a decrease in insulin-stimulated phosphorylation of Akt, thereby suggesting a GnRHR-AAb-mediated impairment of insulin signalling in peripheral tissue via upregulation of insulin-stimulated p-IRS-1(S636/639) and downregulation of insulin-stimulated p-Akt473 in IMM liver and skeletal muscle.

To evaluate instead the hypothesis that natural autoantibodies to the FSHR or LHR might be prevalent in PCOS, Schniewind et al. (522) utilized ad-hoc established luminometric assays based on recombinant human receptor fusion proteins utilising luciferase as reporter to determine the prevalence of FSHR-aAb and LHR-aAb in serum samples from healthy controls and PCOS patients. The analysis revealed a relatively low prevalence, with 2.0% samples positive for FSHR-aAb in the PCOS group and 0.4% samples positive for LHR-aAb, compared to 0.9% and 1.2% respectively in the control group. They concluded that, although the FSHR and LHR do constitute potential autoantigens in human subjects, it is overall unlikely that autoimmunity to the LHR or FSHR constitutes a cause of hyperandrogenemia or ovulatory dysfunction in PCOS.

When analysing an antibody as a potential mediator for autoimmune disease, Witebsky defined three main criteria (551,552): the importance of being able to evaluate a quantitative response from the target tissue, as well as the transferability of the specific condition through the antibody, and stimulation of an exact receptor containing cells. Based on the currently available results no common circulating disease-specific autoantibodies have so far reproduced a sufficiently PCOS-like phenotype, with both metabolic and reproductive features, further underlining the complexity of the disease.

1.6 Animal models for immunological studies in PCOS

To further understand the pathophysiology of PCOS, to isolate specific immunological features being such a multifactorial inflammatory disorder as well as mechanisms of inheritance, how to predict it and finally how to increase accuracy in treatment and prevention, animal models continue to be an important approach.

By definition, nonhuman species cannot exhibit PCOS and there are no veterinarian diagnoses for such condition in animals. Experimental manipulations to induce a PCOS-like phenotype are numerous and include both short and long-lasting alterations for a programmed or activational manifestation of the pathophysiology (101). To be of relevance for PCOS investigation however, animal models need to fulfil the same diagnostic criteria, with the presence of at least two of the three PCOS-like conditions

as described by the Rotterdam criteria (101). A first distinction is made based on manipulations that permanently alter tissue structure and function such as discretely-timed gestational manipulations or genetically manipulated models. Conversely, peripubertal or adult onset alterations commonly subside when the manipulations cease. It is worth mentioning the possibility of developing singular PCOS-like traits among those described by the Rotterdam criterion (101), as well as models that incur in PCOS-excluding endocrinopathies, and finally a branch of manipulations may lead to PCOS-like features while being originating from non-PCOS like mechanisms, all of which need to be considered limiting in their usefulness for any PCOS analysis.

Among the PCOS-like models that fulfil the mentioned requirements there is a wide range of manipulative approaches, from genetically derived mice models (553), hormonally and nonhormonally manipulated models (554,555) and naturally hyperandrogenic nonhuman primate (NHP) models with genetic similarity to humans (556,557) providing certainly more relevance for etiopathogenic studies (101). For the relevance of this dissertation, rodent PCOS-like models will mainly be discussed and will include an overview of the immunodeficient mouse models for recombination-activating genes 1 (RAG 1 KO) as well as the B cell-deficient muMT$^-$ mice, in light of the aim of this study.

Overall, there are two major strategies applicable to produce animal models for the investigation of PCOS pathogenesis. One is via direct treatment with high levels of androgens which can be induced either by exogenous androgen, or by utilizing an aromatase inhibitor to impede androgen conversion to estrogen (558). Another possibility is by fetal hyper exposure of androgens during gestation (559,560) in order to allow transgenerational studies in offspring which develop PCOS-like traits later in life.

Prenatally androgenized (PNA) female rats and mice grow to develop a complete PCOS-like phenotype. PNA models originate from an initial subcutaneous or intramuscular injection of dams with testosterone (T) or DHT, and recently also AMH has been proven its efficacy, at different gestational ages. Currently the most complete and accurate PCOS-like model with a higher BMI phenotype are the NHP models, and along with sheep as habitual mono-ovulators, truly emulate polycystic ovaries. PNA

mice models instead can better isolate the hyoperandrogenic pathological stimulus as they tend to remain rather lean. When combined with different genetic backgrounds, PNA models are possibly the most suited and efficient for studying PCOS molecular pathways (101). The PNA models age with all 3 criteria, combining hyperandrogenism, oligoovulation and PCOM (99,264,561,562). When developed on a AR knockout genetic background, PNA mice do not exhibit excessive circulating androgens nor a PCOS reproductive phenotype, simply confirming that the PNA model is an AR-activation driven phenotype (102).

Another important revelation from prenatal studies is the developmental outcome of excessive exposure of AMH in pregnant mice, as the female offspring present all signs of PCOS-like phenotype (563). These findings corroborate the notion that had already been theorized (563,564) suggesting the potential pathological connection between increased gestational AMH in hyperandrogenic women, particularly at the late stages of pregnancy (563). This seems to be a sensitive time window which would eventually trigger a PCOS-like phenotype in future offspring (564).

Neonatal manipulation have proven ineffective (101), however, peripubertal DHT exposure instead, of mice and rats, has proven to stimulate an adult phenotype with PCOS-like features ranging from endocrine, reproductive, and metabolic (562,565–569) – possibly also exposing related psychiatric (49) – disorders. This model was adopted for this thesis project and will be better described further on. Briefly a subcutaneous implant can maintain PCOS-like features for as long as the DHT is released. This approach has proven to be relatively effective also in adulthood (101).

Among the peripubertal murine models are also the letrozole-induced (558,566,570). Letrozole is by definition an aromatase inhibitor and the concept for this model was developed based on rare CYP19A1 variants, the aromatase gene, which directly hinders aromatase activity (517). Women reporting hyperandrogenism and particularly an ovulatory dysfunction phenotype of PCOS have normal or even higher estrogen levels, which have been compared to the midfollicular phase of a healthy menstrual cycle (571,572). Letrozole-induced adult female mice do not develop metabolic traits (573), representing a lean reproductive PCOS phenotype.

These manipulations have all allowed to better clarify how increased androgen and AR activation affects PCOS development, as well as fetus programing and the uterine environment. Nonetheless, most of the described models have a few intrinsic limitations which are to be taken into account. Firstly, seen as most animals develop obesity, it is therefore difficult to separate the effects of hyperandrogenemia from obesity induced reproductive, metabolic and immunological dysfunction. Furthermore, already before pregnancy, women with PCOS generally exhibit high levels of androgen, implying that oocytes have been already exposed to androgen excess long before fertilization, and lastly, the pharmacological doses of T or DHT introduced after birth or during gestation may not truly reflect the androgen environment of PCOS. Generally, testosterone and DHT levels measured in ovarian follicular fluid and/or serum are 1.5 to 3.9 fold higher in women with PCOS compared to unaffected women (567,574–576).

The latter component was confronted by Xue, P. et al. (577) which developed a protocol for chronic DHT exposure from insertion of a pellet with 4mm length of crystal DHT powder, describing both reproductive and metabolic alterations in an adult mouse model within two weeks of initiation. Main reproductive alterations affect ovulatory cyclicity, which is evaluated based on vaginal cytology (578): predominant nucleated epithelial cells suggest proestrus stage, predominant cornified squamous epithelial cells are typical of the estrus stage, both cornified squamous epithelial cells and leukocytes indicate the metestrus stage, and predominant leukocytes indicate the diestrus stage which is commonly also the longest phase of murine cycle.

This newly developed model has circulating serum DHT levels that are within a 2-fold increase compared to control mice. Interestingly, these DHT-exposed mice have been described as not exhibiting notable alterations of basal serum estradiol, testosterone, LH and particularly do not develop obesity, with similar ovarian weight, serum levels of cholesterol, free fatty acids, leptin, TNFα and IL-6 relative to controls even up to 3.5 months after DHT insertion (579–581). This aspect can really be leveraged to dissociate both the reproductive (e.g. follicle development, ovulation and fertility) and metabolic consequences (e.g. glucose or lipid metabolism, body composition, adipose depot distribution) caused by excessive circulating androgen from those that are related potentially to obesity, allowing for in depth tissue specific assessments. In

addition, the mating of these DHT-exposed females would allow to address the impact of a hyperandrogenic maternal environment both on oocytes and on the health of the offspring (560), addressing the concerns which limit other PCOS-like models.

As described by Xue, P. et al., it is important to note that in order to stably maintain overtime relatively similar levels of androgens to those of women with PCOS, a continual exogenous DHT exposure is required as there is not endogenous programmed hyperandrogenism. Consequently, researchers have noticed a metabolic phenotype from 2 weeks onward or 14 days from DHT exposure, which however subsides or is slightly attenuated after the fourth week or 28 days. Interestingly, no attenuation was observed for reproductive dysfunction. This is particularly relevant, because, as observed by others, chronically exposed models to DHT after birth do not show increased LH pulsatile frequency, which points toward different possible pathological mechanisms of androgen induced reproductive dysfunction which may vary between developmental and late-onset acquired hyperandrogenism.

Finally, customized genetically manipulated mouse models have proven to be decisive in mechanistic studies allowing to isolate specific pathways and developmental phases. Particularly studies combining DHT-induced hyperandrogenic mouse models with complete or cell-specific AR KO as well as with immunodepleted knock out mice models, which allowed for significant advances in understanding the role of multiple AR target sites. For instance, although not within the scope of this dissertation, it is important mentioning how AR inhibition in the brain, particularly in the pituitary region, prevented partial or complete development of key PCOS reproductive and metabolic features involved in lipolysis (103) and ovulatory function (582). Clearly the brain is a crucial site and very central to PCOS pathogenesis development. A similar inactivation of AR activity in granulosa cells alone or theca cells in hyperandrogenized PCOS mice did not equally protective effect (103,580).

As discussed thoroughly in previous sections, the underlying chronic inflammation in PCOS is sustained by diffuse cytokines and multiple adipokines (583), profoundly remodelling the immune system repertoire. Understanding how both B and T cells react in the disease process is a crucial step in deciphering the pathogenesis of inflammatory conditions. B cells can manifest their activity twofold: via the initiation of

autoimmune response and or the induction of tissue lesions by secreting pathogenic autoantibodies (584). Recent research has pointed out how peripheral B cells in PCOS increase both in numbers and in their activity potential (442), although it remains to be clarified how central are B cells to the inflammatory process, and how are they affected by the hyperandrogenic hormonal environment. In questioning a potential autoimmune action of B cells in PCOS, it is relevant to distinguish which inflammatory processes are unique and specific to PCOS and which are obesity-related, as plasma of individuals with obesity have proven to be commonly high in circulating IgG antibodies with potential anti-self-reactivity (585). B and T cell deficient models are ideal to dissect the roles these cells have in various phases of pathogenic immune response, in order to isolate the relative effects of each separate adaptive immune system compartments.

A B^{null} homozygous mutant mouse model lacking mature B cells (common name: *muMt-*) was first developed by Klaus Rajewsky and Werner Muller by targeting the membrane exons of the immunoglobulin μ chain gene, with the intent to better understand the role of IgM in B cell development. This strain was described in the 1991 manuscript by Kitamura et al. (586), both the chimeric and homozygous model, in an attempt to isolate any variation in B cell phenotype. B-cell development in the heterozygous mice seemed to be normal, but in homozygous animal's B cells were absent, revealing a block in B cell development at the level of pre-B cells. Of the various classes of antibodies released by B cells, class M is the first to be expressed on the membrane of the developing cells. In consequence, these events revealed how essential surface IgM appears to be in promoting the differentiation of large pre-B cells toward small pre-B cells, pinpointing the developmental stage when this event occurs. However, although being a well-established engineered technology, B cell deficient models obtained by anti-μ treatment remain an extreme model, and may have unrecognized secondary effects. In consequence, there are still debates about the role of B cells-activated or nonactivated, respectively-in priming of T cells, although their role in stimulating previously primed T cells is widely accepted.

Modulation of the adaptive immune response is based on reciprocal interaction between B cells and T cells. Variations in B cell activity, signalling and development are very often T cell dependent. For example, in obesity-related IR, T cells within the VAT adipose tissue have been found to be limited in their T cell receptor repertoire,

which in turn affects the affinity of antibodies generated by infiltrating B cells, driving an antigen-specific immunity in VAT inflammation (437). In an attempt to discern the role for T cells, the reconstitution of RAG 1 mice models, which lack lymphocytes entirely, can be a useful approach. RAG-1 KO mice were first introduced by Mombaerts and lacomini in 1992. These mice have small lymphoid organs that do not contain mature B nor T lymphocytes. The lack of lymphocytes, both B and T cell is altered at a very early differentiation stage and correlates with the inability to perform V(D)J recombination. The immune system of the RAG-1 mutant mice has been described as that of nonleaky scid mice. This model has quickly established itself as central model in immune studies as no obvious neuroanatomical or behavioural abnormalities have been found in the RAG-1-deficient mice that may suggest any side effects from the germline mutation.

1.7 Hypothesis and Aims

1.6.1 Hypothesis

Inflammation is central in the pathophysiology of PCOS, tightly associated with hyperandrogenemia (1,587). Variations among B cell subsets have been observed and the notion that B cell depletion may have a protective role in PCOS is being suggested (442). Indeed, in vitro assays with B cell effector IgG antibodies demonstrate a dose-dependent stimulatory efficacy on the GnRHR (546). What remains unclear is whether altered immune compartments leading to potential autoimmune conditions are a cause or consequence of hyperandrogenic hormonal milieu in PCOS (357). Based on these premises, we hypothesized that excessive circulating androgens such as in PCOS may pressure B cell development processes, enhancing the production of a proinflammatory repertoire, skewed towards auto reactivity. This thesis thoroughly examined if self-reactive B cells and their effector antibodies have a causal role in the development of these immunometabolic abnormalities by combining both human case-control explanatory studies with an in vivo experimental application for the dynamic study of antibody transfer mechanisms in mouse models.

Taken together, our hypothesis was that high androgen exposure on B cell phenotypes

induces the production of proinflammatory subsets, such as DN B memory cells, and influencing levels of antibody titres.

1.6.2 Aims:

The aim of this thesis was to investigate the role of B cells in PCOS and whether B cells are contributing to the pathology as well as the overall effect of hyperandrogenism on B cell populations.

Specific aims:

1. Characterize main B cell lineages and subpopulations, as well as circulating antibodies in human serum of women affected with PCOS
2. Assess an underlying autoimmunological activity for PCOS-deriving IgG
3. Characterize androgen-mediated regulation of B cell phenotypes in other relevant tissues
4. Examine transmissibility of the disease through B cells
5. Assess whether B cell deficiency may indeed be protective towards developing PCOS-like features

2. MATERIAL and METHODS

2.1 Human study cohort.

Between September 2019 and March 2021, at the endocrinological Outpatient Clinic of the Medical University Clinics in Graz (Austria) 15 women of Caucasian ethnicity were screened for a PCOS diagnosis based on the two main PCOS hyperandrogenic phenotypes. The Phenotype A presents clinical hyperandrogenemia, oligo-anovulation and polycystic ovarian morphology (PCOM), while the Phenotype B relates mainly to a state of clinical hyperandrogenemia and oligo- anovulation without a clear diagnosis for PCOM. The modified Ferriman-Gallwey (mFG) Score was used to asses

clinical hyperandrogenism, based on a self-reported score of eight or higher indicating hirsutism (21). For total testosterone, cut-off was based on a previously published standard of 0.6 ng/mL (2.1 nmol/l), representative for a population sample (431). The diagnosis of oligo-/anovulation was based on menstrual cycles of >35 days or the total absence of menstruation for three or more consecutive months. Medical History was used to assess polycystic ovarian morphology based on a gynaecological ultrasound diagnosis in the participant's medical history. Laboratory analysis were necessary to exclude thyroid disorder, congenital adrenal hyperplasia, Cushing's syndrome, hyperprolactinemia, androgen-secreting tumors, and pregnancy by measuring values of thyroid-stimulating hormone (TSH), 17-hydroxyprogesterone (17OH-P), cortisol, prolactin, as well as clinical examination and pregnancy test. The exclusion criteria took into account multiple factors that may affect participants' immune and hormonal profiles such as neoplastic, infectious, and autoimmune diseases as well as currently used hormonal contraceptives or immunomodulating drugs. 15 volunteers formed the cohort of women affected with PCOS and their fasting blood samples were compared to 22 controls to examine B cell frequencies. We also checked for variations in circulating antibodies (15 PCOS samples and 18 from the 22 controls). In the case of missing values from the final parameters due to laboratory incongruences, participant's results were excluded from the analysis report for that specific variable. All recruitments were undertaken by routine doctors, and nurses involved in the project. Participants were requested to provide a formal oral and written informed consent and all procedures of this project were acknowledged by the Ethics committee of the Medical University Graz (EK 31-560 ex 18/19). Moreover, all experiments here described have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. For the follow-up explanatory studies based on human IgG transfer, 10 women accepted to voluntarily participate in the study during the recruitment phase between February 2020 to October 2020. Seven women were diagnosed with PCOS, of which 1 did not fulfil the overall inclusion criteria and 2 decided to eventually drop out, leaving 4 women with PCOS and 4 healthy donors for the collection of serum for the antibody transfer tests.

2.1.1 Clinical examination, blood sampling, and biochemical measurements.

During clinical examination, anthropometric measures were taken to define body mass index (weight (kg)/height $(m)^2$) and waist-to-hip circumference based on weight, height, waist circumference. Baseline fasting blood samples were drawn for each participant in serum, EDTA, and lithium heparin tubes. Fasting serum samples were analysed for hormonal levels with the following methods: total and free testosterone, androstenedione, and progesterone were measured by liquid chromatography–tandem mass spectrometry based on a previously published protocol (431); sex hormone-binding globulin (SHBG), anti-Müllerian hormone (AMH), and insulin were measured by automated chemiluminescence immunoassay (ADVIA Centaur XP, Roche, Rotkreuz, Switzerland); serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured by enzyme-linked immunosorbent assay (ELISA, DIAsource Immunoassay, Belgium); Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, glucose were measured by automated enzymatic colorimetric assay (Cobas, Roche, Germany). The area under the curve (AUC) for glucose and insulin was calculated from the oGTT using the trapezoidal method. Serum SHBG and testosterone were used to calculate the free androgen index as serum total testosterone/SHBG×100.

2.1.2 Chemiluminescent ELISA.

The assessment of total IgM, IgG and Ig was performed via chemiluminescent ELISA test of human samples as described in previous protocol (588). According to this protocol, by utilizing purified anti-human IgM, IgG and IgA (BD Pharmingen, San Jose, CA, USA) at concentrations of 5 μg/ml in 50 μl phosphate-buffered saline (PBS)-EDTA, a 96-well white, round-bottom microtitration plate (MicrofluorII round-bottom; Thermo, Rochester, New York, USA) was coated and incubated overnight at 4°C. Following a washing and blocking step with a Tris-buffered saline (TBS) solution containing EDTA (pH 7.4, containing 1% bovine serum albumin - BSA) left for 30 min at room temperature, the plate was incubated with plasma samples in their respective dilutions in 1% BSA in TBS with EDTA (pH 7.4) for 2 h at room temperature or overnight at 4 °C. For detection either alkaline phosphatase (AP)-labeled goat anti-human IgM (μ-

chain specific; Sigma-Aldrich, Vienna, Austria; 1: 50,000 in TBS BSA), AP-labeled goat anti-human IgG (γ-chain specific; Sigma-Aldrich, Vienna, Austria; 1: 50,000 in TBS BSA) or AP-labeled goat anti-human IgA (α-chain specific; Sigma-Aldrich, Vienna, Austria; 1: 50,000 in TBS BSA) respectively, were added to the plate. The final detection based on AP-conjugated secondary reagents was performed on a Lumi-Phos (Lumigen, Southfield, Michigan, USA; 33% solution in water) and a Synergy 2 Luminometer (BioTek, Winooski, Vermont, USA). Washing steps were performed on an ELx405 Select Deep Well Microplate Washer (BioTek, Winooski, Vermont, USA) with PBS or PBS-EDTA. Internal controls were included on each microtiter plate to detect potential variations between plates. The intra-assay coefficients of variation for all assays was considered acceptable between 5–15%.

2.1.3 Lymphocyte phenotyping of human samples.

According to an already discussed protocol (589), all human blood samples from the baseline visit were processed within 4 hours for cytometric analysis. The preparation or B-cell phenotyping then consisted in isolation of PBMCs from lithium heparin whole blood by Ficoll gradient density centrifugation. One million PBMCs were incubated with the following antibodies: CD19-VioGreen (clone REA675), anti-IgD-VioBlue (clone IgD26), CD27-APC (clone M-T271), CD86-PE-Vio770 (clone FM95), CD38-FITC (clone IB6), and anti-IgM-PE (clone PJ2-22H3, all purchased from Miltenyi Biotec, Bergisch Gladbach, Germany). Samples were measured using a FACSLyric flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and gating analysis based on FACSSuite (BD Biosciences).

2.2 Animals and study design.

For the present study, all mice were C57BL/6J background females. According to the different experimental research aims, for the transfer of human IgG 24 five-week-old female C57BL/6JRj mice were obtained from Janvier Labs while Rag1 $KO^{-/-}$ were generated by breeding 10 Male and 10 Female B6.129S7-Rag1 (homozygous for Rag1) from breeding pairs purchased at Jackson Laboratory. For the immune characterization of peripubertal DHT-induced PCOS mice, 30 three-week-old female C57BL/6JRj mice were purchased from Janvier Labs and left to acclimatize for 7 days.

For the B cell reconstitution 10 three-week-old female C57BL/6JRj mice were purchased from Janvier Labs in order to develop the peripubertal DHT-induced PCOS model. MuMt$^-$ mutant mice were bred from 10 Male and 10 Female B6.129S2-Ighm (homozygous for Ighm) breeding pairs purchased from Jackson Laboratory.

Mice were all maintained under a 12-h light/dark cycle and in a temperature-controlled room with ad libitum access to water and a diet. Notably, all mice experiments were carried out in compliance with the ARRIVE guidelines and in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 associated guidelines, EU Directive 2010/63/EU for animal experiments. These animal experiments were all approved by the Stockholm Ethical Committee for animal research (20485-2020) based on the Swedish Board of Agriculture's regulations and recommendations (SJVFS 2019:9) and controlled by Comparative Medicine Biomedicum at the Karolinska Institutet in Stockholm, Sweden.

In order to develop the peripubertal DHT-induced PCOS mouse model, a 5 mm silastic implant containing 2,0-2,5 mg of continuously releasing DHT was utilized following a previously published protocol (577). This implant was placed subcutaneously in the neck region of 28-29 days old C57BL/6JRj female mice. Surgery was performed under light anaesthesia and with the use of isoflurane. In a similar manner, control mice were implanted with an empty, blank implant. A third group received in addition to the DHT implant also a 4.5 mm continuously releasing pellet containing 25mg of flutamide (releasing time 90 days, Innovative Research of America, cat. number NA-152), an androgen receptor antagonist which would allow to discern androgen receptor activation effects. Hence, mice were randomly allocated to one of these three groups: control, DHT, DHT-flutamide. The PCOS-like phenotype was considered fully developed only after 3 weeks of exposure. MuMt$^-$ mutant mice were generated from 10 Male and 10 Female B6.129S2-Ighm (homozygous for Ighm) breeding pairs from Jackson Laboratory. No mice received further monthly implants.

2.2.1 Purification and transfer of IgG.

Utilizing a HiTrap Protein G HP purification column (Bio-Sciences AB) IgG was purified from human sera based on the vendor’s instructions. For this protocol, samples were

centrifuged at 3000 RCF for 5 minutes at 4°C and supernatant was diluted 5x with binding buffer. Our final elution containing extracted IgG was dialyzed overnight at 4°C against endotoxin-free PBS and further filtered to obtain sterile antibody solution. To measure exact IgG concentration in each sample we utilized a QUBIT (Thermo Scientific) according to vendor's instructions and stored at -20°C. Samples from each cohort were the pooled separately. The day before injection, the grouped samples were filtered and concentrated using Amicon Ultra-15 Centrifugal Filters, (30kDa MWCO - 15 mL sample volume) according to vendor's instructions (Merck Millipore). For this process the samples needed to be thawed and kept at 4°C the night before concentration. After an initial filtering procedure though a sterile 0.22 µm syringe filter, the desired concentration was reached by multiple spinning at 1000g/4°C and repeated measurements tested by QUBIT (Thermo Scientific) until reaching a final concentration of 4 mg/ml of IgG antibody in a total volume of 450µl of endotoxin-free PBS for injection day 1 and 3, and 365µl at injection day 10. The 7-week-old female C57BL/6JRj mice that were randomly divided into 2 study-groups of 6 mice each, then received the purified human IgG (>98% pure) via intraperitoneal injection (i.p.) in endotoxin-free PBS. Injections were performed as mentioned on days 1, 3 and 10. This exact procedure was repeated utilizing age-matched in-house bred mutant Rag1 $KO^{-/-}$ mice in order to assess the role of T cells mediating the response to IgG.

2.2.2 Assessment of reproductive phenotype.

A first marker of androgen exposure, anogenital distance, was measured at baseline and before sacrifice. More exactly, for the transfer of human IgG, anogenital distance was measured 1 week following the first i.p. injection in both WT and RAG1 $KO^{-/-}$ mice. For the immune characterization, anogenital distance was measured instead 3 weeks after DHT/flutamide implantation. For B cell reconstitution, anogenital distance in reconstituted muMt- mice was measured 2 weeks after the reconstitution. Another assessment of increased androgen exposure, estrous cyclicity variability was assessed by daily vaginal smear for twelve consecutive days (three ovulatory cycles).

2.2.3 Assessment of metabolic phenotype.

Body weight was recorded weekly, while the exact body composition was assessed by magnetic resonance imaging (EchoMRI-100 system, Houston, TX, US) in order to assert the total fat and lean mass. For glucose metabolism, an oral glucose tolerance test (oGTT) was performed after a 5-hour fast. For this procedure, mice received 2 mg per gram body weight of D-glucose (20% glucose in 0.9% NaCl) administrated by orally by gavage. Blood glucose was then measured at baseline and repeated for 4 consecutive time points, at 15-, 30-, 60- and 90-min following glucose administration (Free Style Precision). Blood collection was through tail bleeding into EDTA coated capillary tubes and at 15 min for insulin measurement. Plasma was separated by spinning the samples at 2000 g for 10 minutes at 4°C and stored at - 20°C. Based on the study design for individual project objectives, for the transfer of human IgG as well as B cell reconstitution, we opted to assess first in mice metabolic variations and oGTT when the expected effects from the transfer on glucose metabolism were most likely at their peak, followed by Echo MRI evaluation. For project characterization of DHT-induced PCOS-like mouse model as well as the characterization of androgen exposed muMt- mouse model, mice were first screened through EchoMRI to measure total fat and lean mass and following an oGTT evaluation.

2.2.4 Biochemical assessment of insulin and sex steroids in mice.

Plasma insulin from oGTT was analysed by an ELISA kit (Crystal Chem). Testosterone, androstenedione, estradiol, estrone, and progesterone were measured in serum using a high-sensitivity liquid chromatography–tandem mass spectrometry assay based on a previously published protocol (590).

2.2.5 Tissue collection and cell isolation.

Based on the different experimental timelines, mice were sacrificed at a preadolescent stage, except for immune characterization of the peripubertal DHT-induced PCOS model for which two different time points were considered. The exact timing for each study group were as following: for the transfer of human IgG, C57BL/6JRj WT mice were sacrificed at 13-14 weeks of age. Rag1 $KO^{-/-}$ receiving human IgG were sacrificed

at 16-17 weeks of age. As mentioned, for the immune characterization of the peripubertal DHT-induced PCOS model, we conducted two independent experiments to evaluate separate time points: a first cohort of C57BL/6JRj mice were sacrificed at 20-22 weeks of age, while in a following assessment DHT-exposed C57BL/6JRj mice were sacrificed at 13-14 weeks of age. For the reconstitution of B^{null} muMt$^-$ mice with splenic B cells following DHT exposure, a cohort of C57BL/6JRj mice were sacrificed at 8 weeks of age, 4 weeks after DHT implant, for the retrieval of spleen B cells. The B-cell reconstituted muMt$^-$ mice were sacrificed at 11-12 weeks of age. For characterization of DHT-exposed MuMt$^-$ model, mice were sacrificed at 13-14 weeks of age. The ovulatory stage of each mice was taken into account, by vaginal smears less than 2 hours' prior sacrifice in order to assure a metestrous or diestrous for all cohorts. Mice were fasted for 2 hours and anaesthetized with isoflurane (Isoflo vet, Orion Pharma Animal Health). Blood was drawn by cardiac puncture using a 21G needle; an aliquot of 150 µl was directly transferred to EDTA coated tube and placed on ice for FACS analysis. Microvette capillary tubes (Sarstedt) were used for serum separation. After dissection, tissues were separated and kept on ice in their respective solutions based on in-house optimized protocols: spleen and lymph nodes in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS); ovaries, endometrium, and VAT tissues were maintained in RPMI containing 2% FBS on ice for cell isolation. For analysis of sex steroid, serum in aliquots of 250 µl was separated by centrifugation at 5000 G for 10 min at 4°C.

2.2.6 Comprehensive B lymphocyte phenotyping of mice tissues.

For the isolation of single cells, spleen along with inguinal and retroperitoneal lymph nodes were directly sieved through a 100 µm cell strainer and centrifuged at 300 RCF at 4℃ for 5 min. For spleen tissue, erythrocytes were haemolysed in 1 ml red blood cell lysis buffer (RBC lyse buffer; 0.16 M NH4Cl, 0.13 mM EDTA and 12 mM NaHCO3 in H2O), followed by a wash in 2 ml of FACS buffer (x2 the volume of RBC lysis). After a centrifugation at 300 RCF at 4℃ for 5 min, all tissue cells were resuspended in flow cytometry buffer (2% fetal bovine serum and 2 mM EDTA in PBS).

Ovarian and uterus tissues were allowed to digest in 1ml and 3 ml of in-house optimized digestive mix (1 mg/ml collagenase type I from 210U/mg, 0.8U DNase I, RPMI, 2% FBS) respectively, while being minced by fine scissors and digested by gentle shaking for 15 and 20 min respectively at 37℃. To inactivate the enzymatic activity, 2 ml and 6 ml respectively of cold flow cytometry buffer was added to ovaries and uterus and placed on ice. Finally, tissues were grinded through a 100µm cell strainer. Samples were spun at 1000 G for 7 min at 4°C and resuspended in flow cytometry buffer.

In a similar manner, visceral adipose tissue was also minced by fine scissors in 5 ml in-house optimized digestive buffer based on RMPI containing 2% of FBS and 1 mg/ml collagenase type IV (type D, 0.15U/mg). A gentle shaking allowed for better digestion for approximately 20-25 min at 37°C. To inactivate the enzymatic activity, 10 ml of cold flow cytometry buffer was added and all tissues were placed on ice before filtering suspensions through 100µm filter and further spinning at 500 G for 5 min. The resuspended cell pellet was left for 30 seconds at RT in 500µl RBC lyse buffer, then finally washed in 1 ml of FACS buffer (x2 the volume of RBC lysis) and centrifuged at 500 G for 5 min at 4 °C.

A volume of approximately 120 µl of whole blood was placed twice in order to allow for consecutive lysis in 1 ml of room temp RBC lysis buffer (for an approximate proportion of 1:10) for 5 and 2 minutes respectively, each time diluted in 2 ml of flow cytometry buffer and spun at 380 G for 5 min at 4°C.

All cells gathered from each individual tissue were then plated on 96-well round bottom plates and stained (Sarstedt, 83.3925.500) first with FC-blocking antibody surface antigen staining (CD16/32, clone 2.4G2, BD Biosciences) diluted 1:100 in flow cytometry buffer, followed by incubation with the chosen antibodies for this study: IgD-Pacific Blue (clone 11-26c.2a, BioLegend), CD19-BV480 or PE/Cyanine7 (clone 1D3, BD Biosciences or clone 6D5, BioLegend respectively), CD45R/B220-FITC (clone RA3-6B2, BD Biosciences), CD21/CD35-PE-CF594 (clone 7G6, BD Biosciences), CD138-PE/Cyanine7 (Syndecan-1, clone 281-2, BioLegend), CD27-APC (clone LG.3A10, BD Biosciences), IgM-APC/Cyanine7 (clone RMM-1, BioLegend), CD86-BV510 (clone GL1, BD Biosciences). Samples were measured using a FACS Canto II

flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were analysed using FlowJo (BD Biosciences).

2.2.7 Total antibody quantification in plasma by ELISA.

To quantify total IgM, IgG1, IgG2b, IgG2c, IgG3 and IgA antibodies in plasma we utilized a chemiluminescent ELISA based protocol as described elsewhere (591). In brief, 96-well white round-bottomed MicroFluor microtiter plates (Thermo Lab systems) or immunoGrade, 96-well, PS Standard plates (781724; Brand) were coated with the respective antibodies: anti-mouse IgM (Sigma; M8644; at 2 µg/ml), anti-mouse IgG1 (Biolegend; RMG1-1; at 2 µg/ml), anti-mouse IgG2b (BD Biosciences; R9-91; at 3 µg/ml), anti-mouse IgG2c (STAR135; at 1 µg/ml), anti-mouse IgG3 (BD Biosciences; R2-38; at 4 µg/ml) or anti-mouse IgA (BD Biosciences; C10-3; at 3 µg/ml) in PBS and left overnight. After washing three times with PBS and blocked with a Tris-buffered saline solution containing 1% BSA (TBS/BSA) for 1 h at room temperature. Wells were then washed with either PBS (plates for IgM, IgG2b and IgG2c) or PBS supplemented with 0.05% Tween (plates for IgG1, IgG3 and IgA), and diluted mouse plasma was added in TBS/BSA to the wells and incubated once more overnight at 4 °C. A final wash preceded the adding of respective anti-mouse IgM antibody conjugated to alkaline phosphatase (Sigma; A9688), the biotinylated forms of anti-mouse IgG1 (BD Biosciences; A85-1) or anti-mouse IgG2b (BD Biosciences; R12-3), anti-mouse IgG2c (JIR 115-065-208), anti-mouse IgG3 (BD Biosciences; R40-82) or anti-mouse IgA (BD Biosciences; C10-1). Wells were washed again as described above and neutravidin conjugated to alkaline phosphatase was added where required. Once again, wells were washed and rinsed once with distilled water, and 25 µl of a 30% LumiPhos Plus solution in dH2O (Lumigen Inc.) was added. After 75 min, the light emission was measured with a Synergy 2 luminometer (BIO-TEK) and expressed as RLU per 100 ms.

2.3 Statistics.

Softwares used for statistical evaluation were either Prism version 9 (GraphPad Software, Boston, MA) or SPSS version 28 (IBM, Armonk, NY, USA) depending on the analysis. Furthermore, all continuous data were first screened for normality by Shapiro-

Wilk test and equality of variance. Normally distributed data were then compared using unpaired Student's *t*-tests. The differences between more than two groups were determined by an ANOVA followed by Tukey's post hoc test. Any differences were considered statistically significant at a $P<0.05$. One individual patient or one animal was considered a biological replicate. In the case of missing values, patients or animals were excluded from the analysis for that variable. The sample size of the human monocentric case-control explanatory study was calculated considering the distribution described in literature associated with specific PCOS phenotypes A and B. In fact, more than half of PCOS patients identified within clinical settings generally present a phenotype A, whereas the other three phenotypes (i.e., B, C, and D) have an almost equal prevalence (592), and taking into account that the presence of hyperandrogenism and BMI (592), and degree of menstrual irregularity (593), even in the presence of no evidence for polycystic ovarian morphology (594), are all independent predictors of metabolic dysfunction.

Hence, with the intention to evaluate the potential effects of double negative autoreactive B cells and assuming from the literature a predicted variation of 10% among total $CD19^+$ B cell populations in PCOS patients based on previously reported assessments (442), a total final cohort of 40 subjects would achieve the minimum required 90% power to define significant differences between the means versus the alternative of equal means using an F test with a 0.05 significance level. An effect size of $f = \sigma m/\sigma$, calculated as 0.31, represented the size of the variation in the means. Sample size was generated using PASS 15.0.6. For animal studies, no statistical methods were used to predetermine sample size. Animals were allocated to experimental groups arbitrarily without formal randomization. Investigators were not formally blinded to group allocation during the experiment.

3. RESULTS

Hyperandrogenemia in PCOS is associated with higher B cell number and immunoglobulin M titres

Similar studies already identified alterations in overall B cell populations when compared to control groups (442), so our first step was to characterize the major populations and subsets based on the marker CD19. All 15 PCOS participants completely fulfilled all three Rotterdam Criteria, having alongside elevated total triglycerides and low HDL-cholesterol values (**Appendix Table 1**). These women were also slightly younger than the 22 healthy controls, being the median ages of 26 and 36, respectively. No other significant anthropometric differences were registered between the groups in an attempt to provide homogenous study cohorts.

We proceeded to phenotypically analyse B cell subsets classifying them based on development and functional activity, using markers IgD and CD27 we confirmed a significant remodelling of all B cell compartments among women with PCOS.

Interestingly, age-associated DN B memory cells lacking surface expression of CD27 and IgD were indeed higher in the PCOS group (**Fig. 1a**), although when analysing antibody secreting potential (CD38 - **Fig. 1e**) and activation (CD86 - **Fig. 1f**) we did not find any evidence of ongoing activity. Unswitched double positive $CD27^{+}IgD^{+}$ B cells, the “innate-like” were lower in numbers (**Fig. 1b**) and values among naïve B cells did

not vary (**Fig. 1c**). Lastly, switched CD27⁺ IgD⁻ were also elevated (**Fig. 1d**) which may impact the frequencies of unswitched as their development is very much interrelated.

Finally, circulating immunoglobulins M (IgM) shown to be elevated in women with PCOS (**Fig. 1g**) among the multiple antibody titres tested (**Fig. Appendix 1a-b),** and tightly linked with their androgen scores ((**Fig. 1h-i**) compared to controls with similar BMI (**Fig. 1j**)

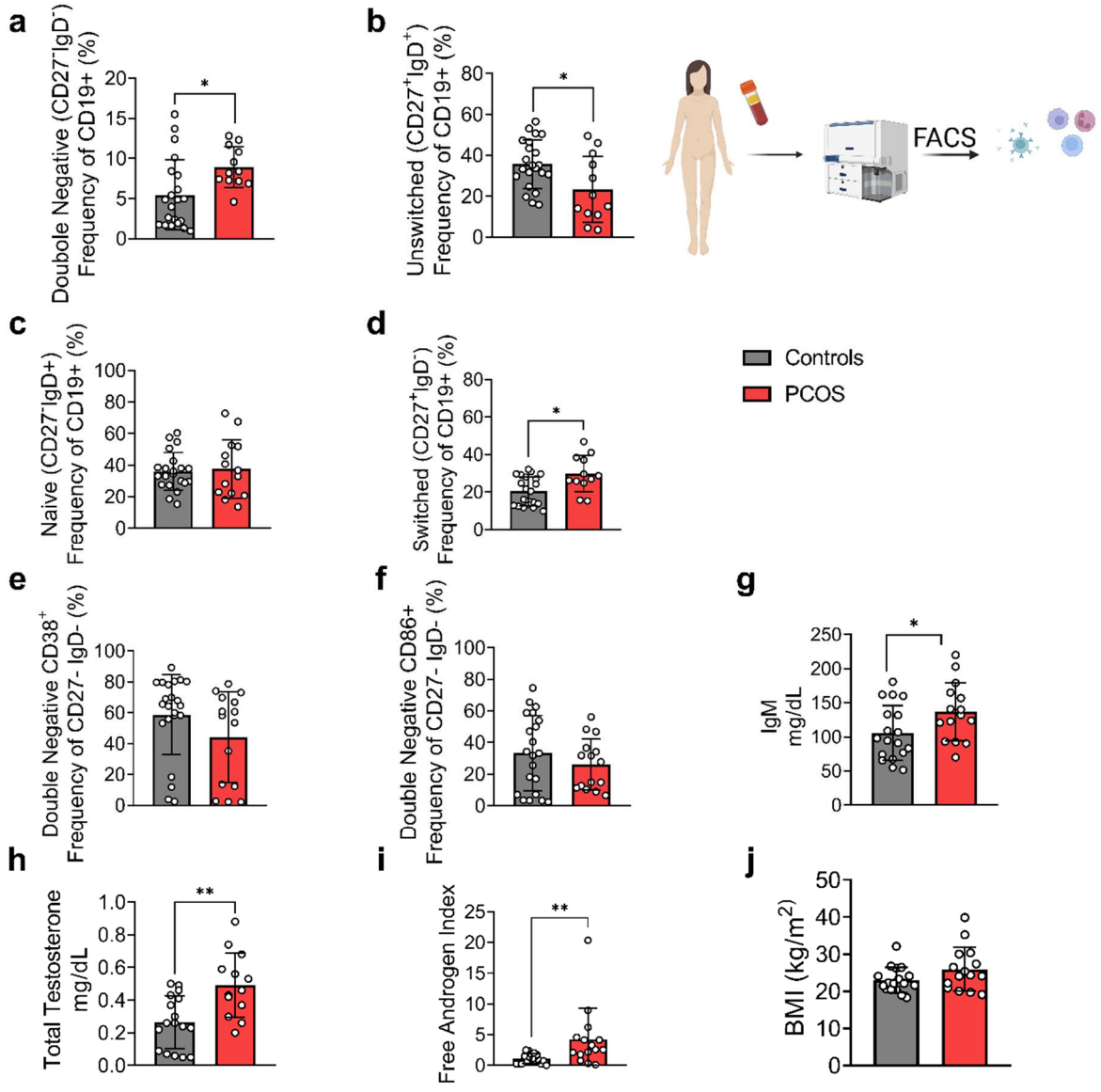

Figure 1: B cell frequencies and immunoglobulin M variations in women with Polycystic Ovary Syndrome.

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Our first assessment supports previous findings of rearranged B cell compartments in PCOS correlating to the presence of higher androgen and abnormal values of circulating IgM antibodies. Although there were no differences in proportion of circulating IgG indicating an inflammatory activity, in these women, clinical markers of lipid metabolism pathways are affected (**Appendix Table 1)**.

IgG antibodies purified from human serum induce an increase in body weight of WT recipient female mice

Age-associated double negative (DN) $CD27^{-}IgD^{-}$ B memory cells are a heterogeneous population, among which certain clusters with pro-inflammatory features can develop plasma cell traits and release higher amounts of IgG on a per cell basis compared to switched memory B cells (489).

For this purpose, with the intention to investigate a potential autoimmunological effector component among PCOS deriving B cells, we tested the inflammatory activity of PCOS-derived IgG. This immunoglobulin was extracted and purified from a cohort of volunteers diagnosed with a classic PCOS phenotype (**Table Appendix 2**). The antibody was then pooled and intraperitonally (i.p) injected into wild-type (WT) mice. In a similar manner, IgG was derived from serum of hormonally healthy women and transferred intraperitonally to a similar age and weight-matched WT mice. At baseline ovulatory cycles did not vary between cohorts (**Fig. 2a-b**). After three weeks following the IgG transfer, the cohort of PCOS-IgG receiving mice showed a significant increase in body weight compared to their controls (**Fig. 2 c**). When assessing body composition however, there was not a major disparity in the proportion of either fat or lean mass between the groups (**Fig. 2d**).

When investigating immune cell populations, we found an interesting effect from the IgG transfer, which seemed to induce variations also among B lymphocytes populations in blood, ovary, and visceral adipose tissue (VAT). Notably values of circulating DN B memory cells were higher (**Fig. 2e**) while blood naïve cells were reduced (**Fig. 2f**), resembling the B cell arrangement previously detailed from blood results of the human donor PCOS cohort. More specifically, among the DN B cells, the DN1 CD21^{+} subset resulted as the main circulating subpopulation in the blood of PCOS-IgG receiving mice (**Fig. 2g**). In the VAT tissue, we found higher frequencies of effector IgM^{+}IgD^{+}CD27^{+} "double positive" unswitched B cells (**Fig. 2h**) while the

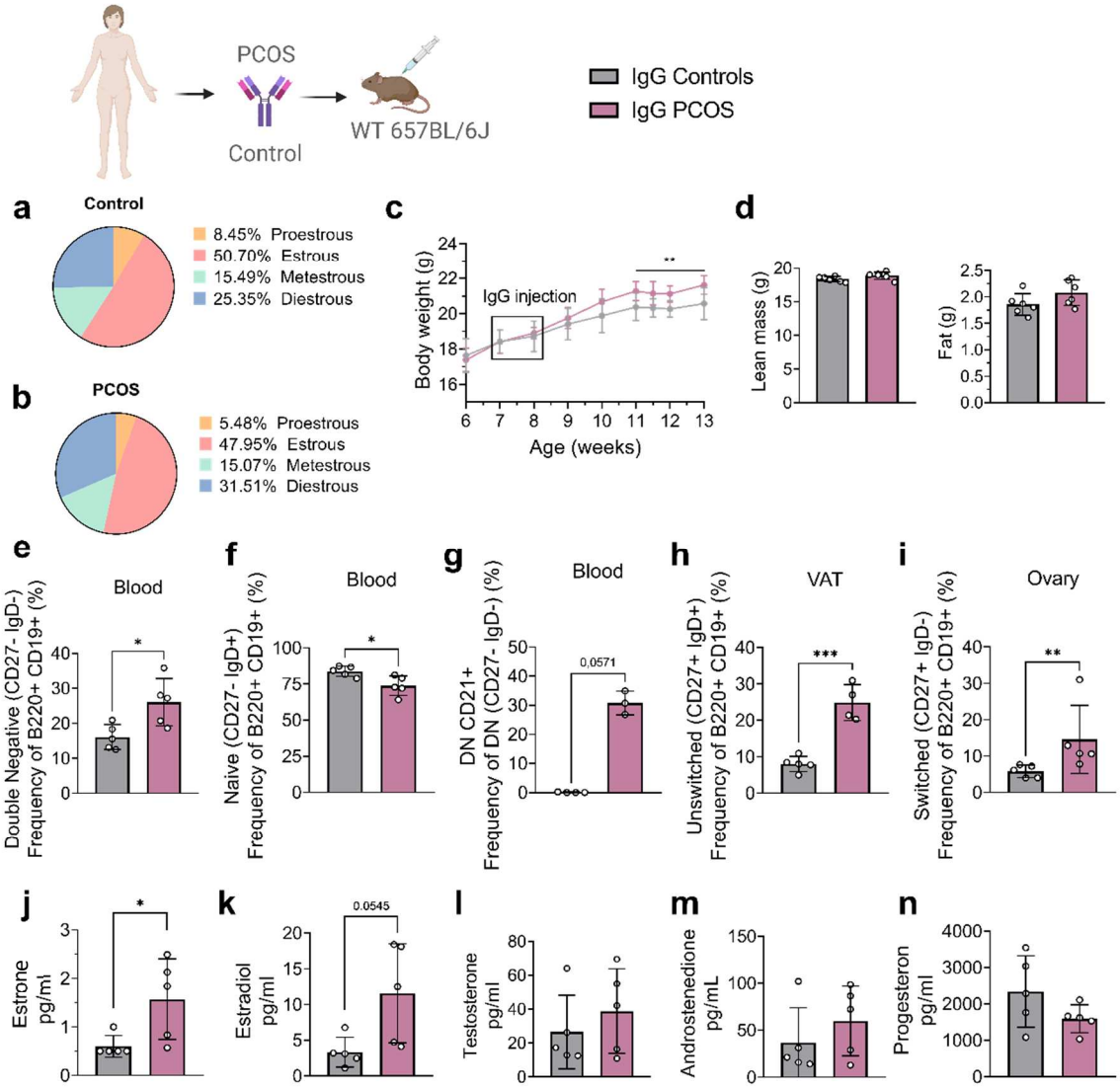

Figure 2 IgG Transfer to WT mice.

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activated switched $IgM^{+}IgD\text{-}CD27^{+}$ were increased in ovarian tissue (**Fig. 2i**). We found interesting variations also in sex steroid values, higher estrone (**Fig. 2 j**) and a trend of higher estradiol (**Fig. 2 k**). Androgens and progesterone were unaffected (**Fig. 2 l-n**).

Being B cell functions directly influenced by T cell populations, we questioned whether the immune modulation associated with weight gain among B cells may be T-cell dependent. For this reason, following a similar procedure human IgG deriving from PCOS and control cohorts, both purified then pooled were separately transferred into 10-week-old Rag1 $KO^{-/-}$ mice, which lack of mature T- and B-cells. Nonetheless, three weeks after the i.p. IgG transfer, all RAG1 $KO^{-/-}$ mice did not develop any PCOS-like phenotype, differently from the previous transfer into WT mice. This points out the necessary involvement of other immune components, as while B cells may certainly drive PCOS alterations that affect energy metabolism and fat deposition, they do not seem to operate alone.

DHT-induced PCOS-like mouse model presents fluctuations in B cell compartments in reproductive, metabolic and immunological tissues.

For our next aim, to investigate the overall effect of androgen exposure of B cell phenotypes and tissue specific variations, we choose to address this question using a well-established peripubertal DHT-induced PCOS mouse model (101). By implanting a subcutaneous pellet with 4mm pellet containing DHT, this model develops into a lean PCOS-like phenotype (577), allowing to focus more specifically on androgen-induced alterations. Controls received a blank pellet, while in a third group was added a second pellet along with DHT, an androgen antagonist, flutamide. This would allow to isolate which variations are AR activation dependent. This experiment was repeated separately, in order to asses different age time points, at 13 and 16 weeks of age respectively.

After 4 weeks post pellet insertion, androgen exposed mice present arrested estrous cycles, generally in either diestrus or metestrus being the longest stages in mice (**Fig. 3a-b**), and longer anogenital distance (**Fig. 3d**). The concurrent treatment with AR antagonist inhibited progression of reproductive symptoms (**Fig. 3c-d**). Although

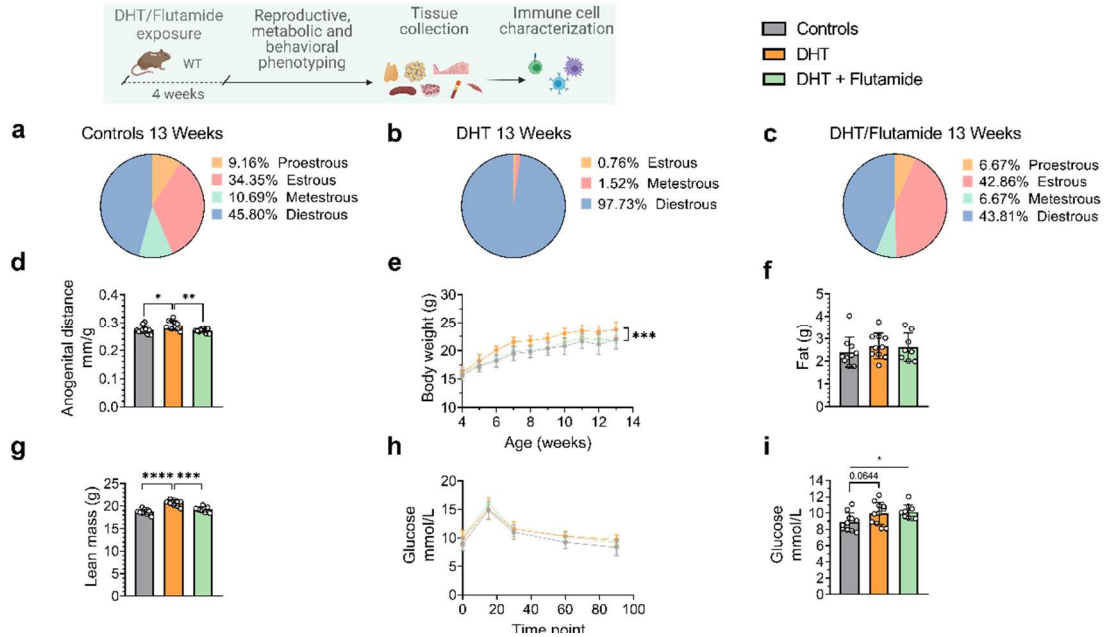

Figure 3 : DHT-induced PCOS-like mouse model phenotypic study at 13 weeks of age

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remaining a lean model, DHT-exposed mice tend to gain more weight than controls (**Fig. 3e**) overall, both fat (**Fig. 3f**) and lean mass (**Fig. 3g**). An oral glucose tolerance test (oGTT) did not underline any metabolic alterations based on glucose homeostasis compared to controls (**Fig. 3h**), rather a trend already at 13 weeks of age of higher fasting glucose (**Fig. 3i**).

DHT-exposed mice analysed at the second time point, 16 weeks of age, proved to have a similarly arrested estrous cycle, the first symptom of a PCOS-like phenotype (**Fig. 4a**), once again either in diestrus or metestrus (F**ig. 4b**). These mice presented also longer anogenital distance (**Fig. 4d**). As in the previous experimental groups, the androgen mediated effects were overall either mitigated or completely inhibited by co-treatment with flutamide (**Fig. 4c-d**). Mice exposed to continuous DHT commonly present higher body weight (**Fig. 4e**) which is mainly lean mass (fat mass **Fig. 4f**; lean

mass **Fig. 4g**). When challenged with an oGTT, mice have irregular glucose homeostasis (**Fig. 4h**) with higher fasting glucose state compared to controls (**Fig. 4i**).

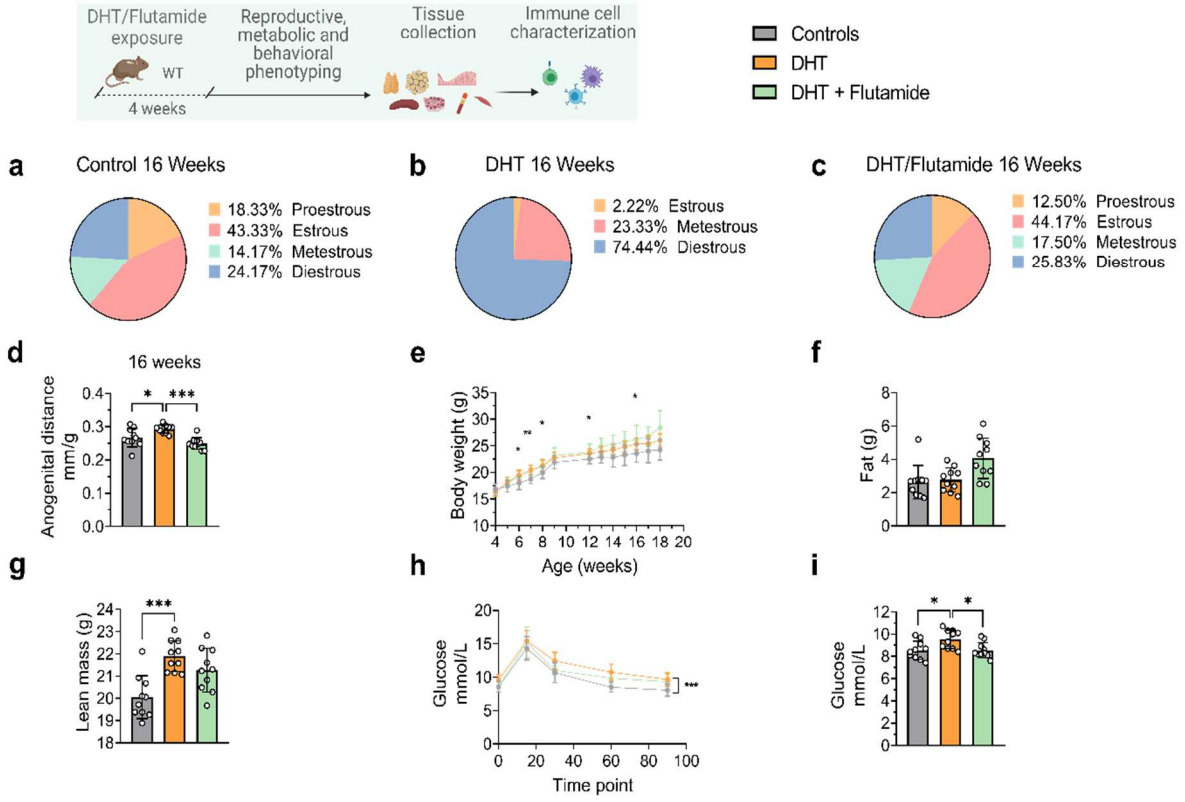

Figure 4 : DHT-induced PCOS-like mouse model phenotypic study at 16 weeks of age

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When assessing B cell distributions, 13-week-old DHT-exposed mice presented variations in multiple tissue, with blood being the most affected. Differently from human tissue, circulating DN memory B cells were lower compared to controls (**Fig. 5a**) while naïve B cells increased in blood (**Fig 5b**). Between 16 and 20 weeks of age, we found the highest variation of DN B cells in the spleen tissue (**Fig. 5c**) with frequencies of naïve B cells still decreased (**Fig. 5d**). Adjunct treatment with flutamide reversed all effects. At 16 weeks of age, it is rather the ovarian tissue that exhibits the most prominent variability from DHT exposure. DN and naïve B cells are both reduced (**Fig 5e-f**), with a significant increase of $IgM^+IgD^+CD27^+$ unswitched B cells (**Fig. 5g**). When assessing for principal DN clusters, we noticed a trending increase DN $CD21^+$ populations in the DHT exposed ovaries compared with controls and mice co-treated

with flutamide (**Fig. 5h**). Incidentally, this specific pattern was observed in blood tissue of mice receiving human PCOS-IgG. Naïve B cells presented similar distribution, expressing primarily $CD21^+$ in ovary (**Fig. 5i**), endometrium (**Fig. 5j**), spleen (**Fig. 5k**) and VAT (**Fig. 5l**). These dissimilarities in frequencies were reversed by co-treatment with flutamide.

Systemic inflammation developed in these models with a PCOS-like phenotype is associated to tissue specific B cell frequencies as a result of AR activation, showed clearly by the adjunct protective effect of flutamide. The evident differences among single tissues would require further experimental analysis.

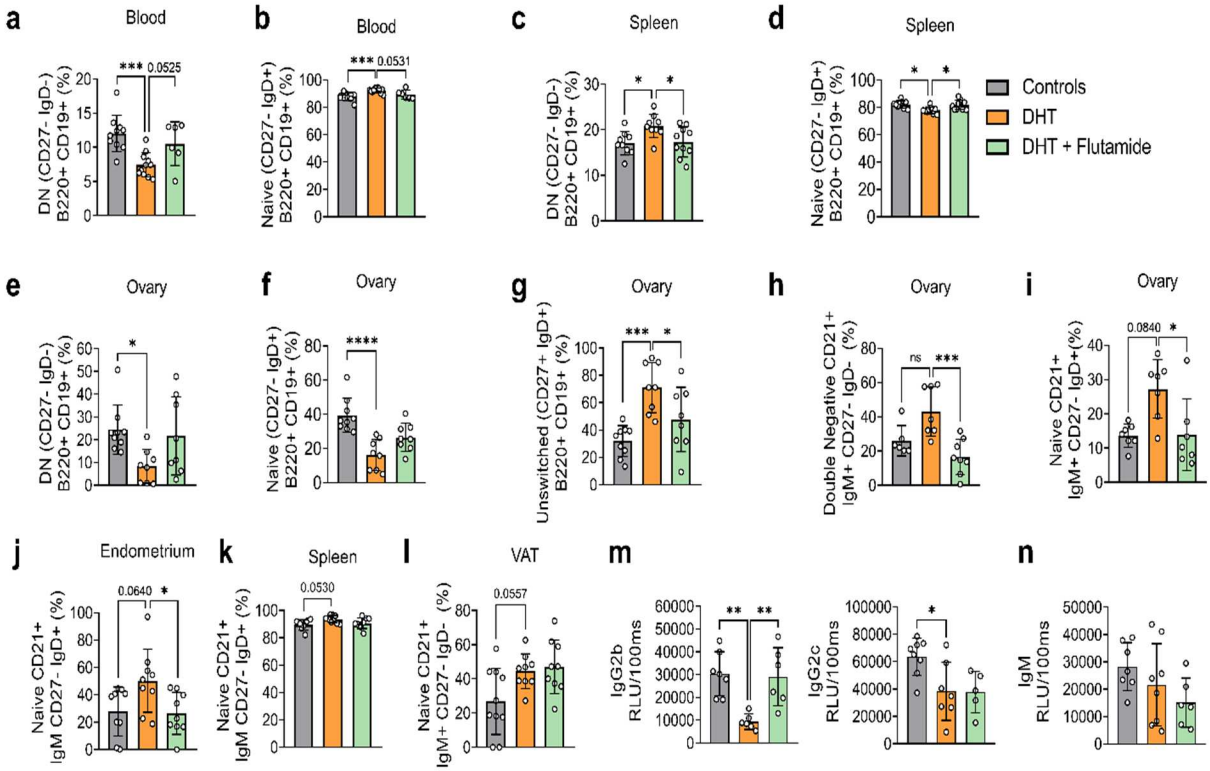

Figure 5 : DHT-induced PCOS-like mouse model B cell frequencies

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Unique IgG profile in DHT-induced PCOS-like mice

The function of B cells, in addition to T-cell interactions, is the production of effector molecules such as antibodies. Having found significant alteration in the titre of circulating IgM in women with PCOS, we questioned whether similar variations were reproducible also in the peripubertal PCOS-like mouse model.

Hyperandrogenic DHT-exposed mice, have reduced levels of IgG2b and IgG2c isotypes (**Fig. 5m**), with no major fluctuations in IgM levels (**Fig. 5n**).

The transfer of B cells from DHT-induced PCOS-like mice does not compromise immune or metabolic processes in recipient mice.

To confirm and isolate the pathological activity of B cells in the development of PCOS from the direct activation of AR, we questioned whether the reconstitution with isolated DHT-exposed B cells would induce a PCOS-like phenotype in recipient muMt$^-$ mice, lacking mature B cells. For this purpose, splenic B cells from DHT-exposed mice were transferred i.p. in to 6-week-old muMt$^-$ B^{null} mice. These mice maintain an active and functional T cell compartment. Similarly, we transferred control muMt$^-$ mice with splenic deriving B cells from control donor group. The DHT-exposed B cells recipient muMt$^-$ mice did not develop PCOS-like traits within the 2 weeks following B cells reconstitution. Overall estrous cyclicity was not affected (**Fig. 6 a-b**), neither was anogenital distance (**Fig. 6c**). There were no variations in body weight (**Fig. 6d-e**). Fasting glucose was not affected (**Fig. 6f**) nor was glucose tolerance in oGTT testing (**Fig. 6g**). These results collectively suggest that the development of a PCOS-like phenotype may not be asserted uniquely to the exposure of B cells alone to a hyperandrogenic environment and seems to require other peripheral mechanisms. Indeed, the continuous stimulus from excessive circulating androgens affects function and properties of other immune cells which could be involved in the disease related abnormalities.

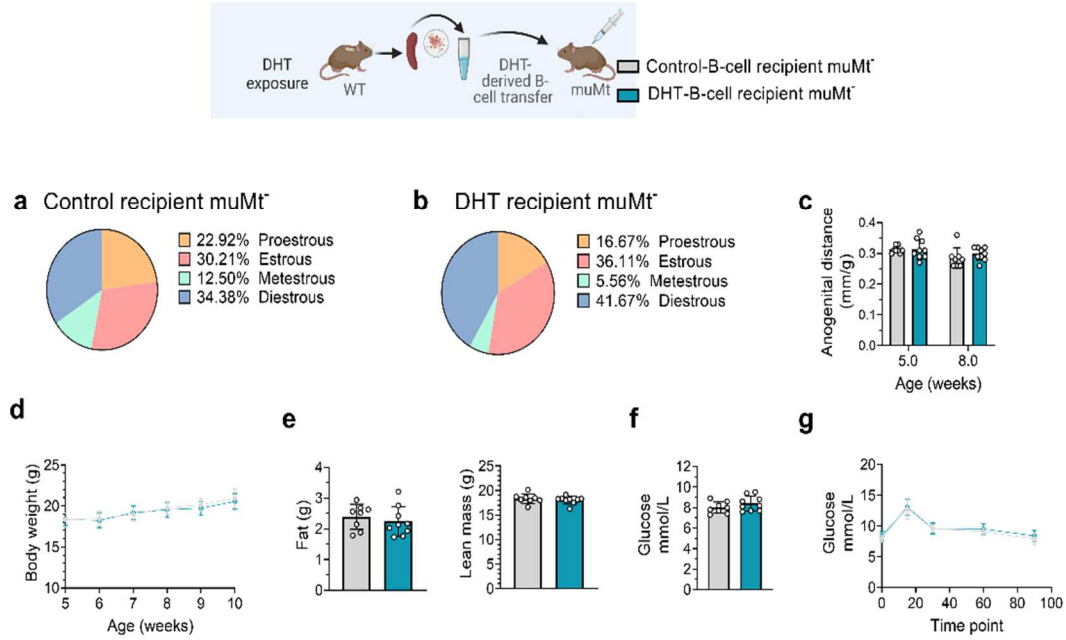

Figure 6 : B cell transfer from DHT-induced PCOS-like mice into recipient muMt- B cell-deficient mice

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B cells deficiency is not a protective factor from developing a PCOS-like phenotype from DHT exposure.

Lastly, to understand whether absence of B cell may grant a protective effect from developing PCOS-like reproductive and metabolic alterations, muMt$^-$ mice 28 days/4-weeks of age were implanted with a silastic implant containing continuously releasing low-dose DHT. Similarly, control mice received a blank implanted pellet. After four weeks from implantation, DHT-exposed B^{null} muMt$^-$ developed a reproductive PCOS-like phenotype, defined as disrupted estrous cycle (**Fig. 7a-b**) all in the diestrus phase, along with longer anogenital distance (**Fig. 7c**). While commencing both as homogenously balanced cohorts at implantation (**Fig. 7d**), muMt$^-$ mice exposed to DHT gained higher body weight compared to controls already after one week following implantation (**Fig. 7e**), with increase both in total fat and lean mass (**Fig.7f**). These mice developed as well a clear metabolic dysfunction with impaired glucose

homeostasis following a oGTT (**Fig. 7g**) higher blood glucose score 90 minutes after administration compared to control (**Fig. 7h**).

From these data we can speculate that although affected in their function, B cells may not be central mediators of PCOS dysfunctions. DHT-exposed B^{null} muMt^{-} seem to rather develop a worsened state with increased adipose tissue which remained generally unaltered in previous experimental models, and is a risk factor for increased disease severity.

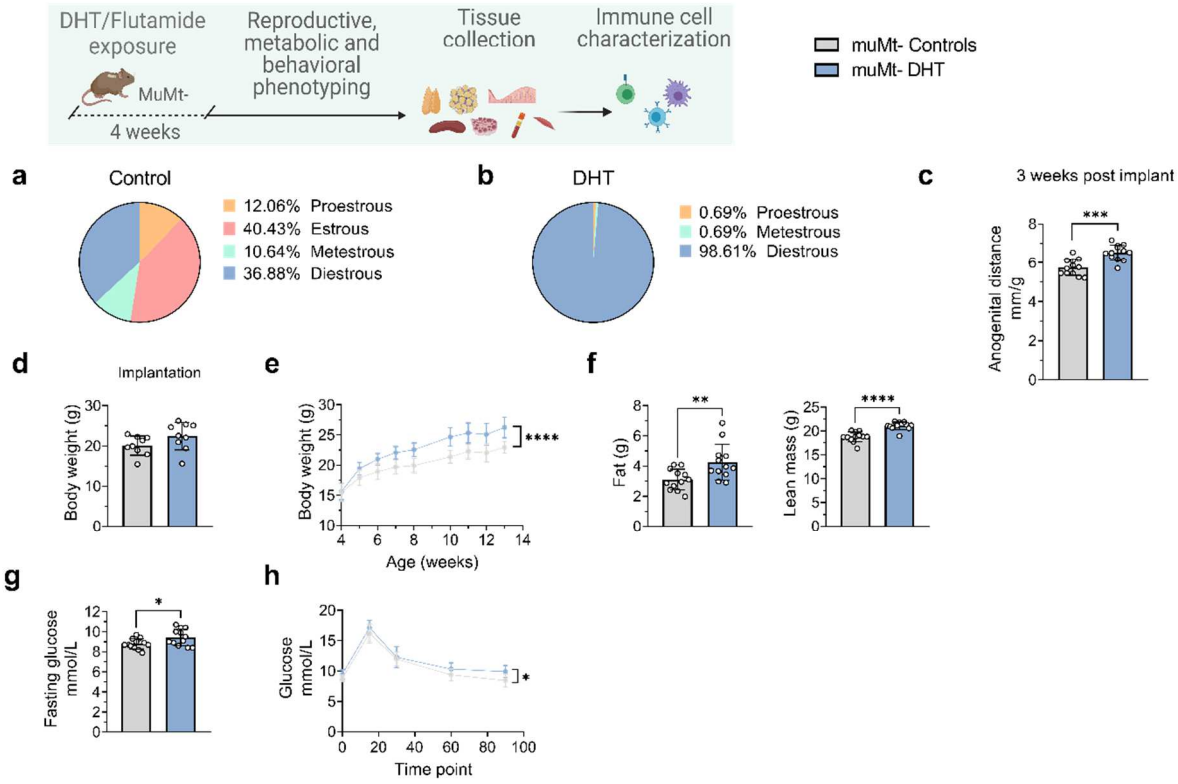

Figure 3 : MuMt- DHT-induced mouse model phenotypic study

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4. Discussion

The present thesis study demonstrated how hyperandrogenemia and altered B cell frequencies as well as antibody titres in a cohort of women affected with PCOS are intrinsically linked, inducing systemic effects through the effector IgG antibodies and resulting in metabolic alterations which lead to a higher body weight as demonstrated by the transfer of human IgG to recipient mice.

We aimed to get deeper insight into the regulatory effects of AR activation, proving how these mechanisms which lead to inflammation and tissue specific immune variations are indeed AR regulated. We believe this may be indeed the first study to describe the effects of AR activation on B cell inflammation in PCOS. We also demonstrate that the lack of B cells does not prevent mice from developing a PCOS-like phenotype once exposed to DHT. Not only was the absence of B cell lymphocytes not protective, but DHT-exposed muMt$^-$ mice produced a stronger inflammatory phenotype aggravated by fat mass accumulation.

The first step was to describe the various B cell subsets and their distinct frequencies, as previous studies have already noticed increased levels of circulating BAFF combined with overall higher circulating B cell numbers in PCOS (442). Our findings highlighted a distinct increase in circulating DN B memory cells which cluster separately from naïve B cells following downgrading of IgD isotype. These heterogeneous tissue-based B cells are associated with immunosenescence, and may also drastically increase during inflammatory conditions (577,595). Among them, distinct clusters can further develop plasma cell properties (489). When assessing T cell co-stimulatory capacity (CD86), and antibody secretion potential (CD38), we did not observe any ongoing proinflammatory activity deriving from DN subset which could support the notion of these cells being a driving force of the underlying chronic inflammation.

PCOS-deriving DN B memory cells resulted being IgM-positive. From previous research on IgM-only cells, this B cell isotype is fundamentally a pool for switched-memory B cells development (596) which are intrinsically poised to plasma cell differentiation upon reactivation (597). PCOS may very well represent a unique

hormonal milieu creating prolonged pressure on the immune system, increasing susceptibility to secondary triggers of inflammation. In line with this theory, we did observe in women with PCOS lower frequencies of unswitched $CD27^+$ IgD^+ memory B cells. In our study, we also noticed a correlation between serum IgM titres and androgen levels, independent of BMI values. In summary, accumulation of adipose tissue particularly in visceral area is indeed a crucial factor leading to chronic inflammation however, we need to acknowledge that, as demonstrated, PCOS does trigger a specific antigenic stimulus which causes a shift in the distribution of humoral immune components.

Autoantibodies targeting histone protein subunits and anti–double-stranded DNA have already been reported in PCOS, although these studies are limited and they remain poorly understood in the context of PCOS (521). The GnRH receptor was truly the first potential target of PCOS-derived antibodies which actually demonstrated in-vitro does-dependent reactivity to PCOS-IgG (598). Based on these observations, in our study we attempted to transfer human PCOS deriving purified and concentrated serum IgG antibodies, which exhibited potential obesogenic action in recipient mice. However, there weren't other fundamental PCOS-like features noticed, such as ovulatory cycles disruption, a universal marker for primary PCOS diagnosis. Interestingly, we did notice an immediate effect on the immune system, with alteration in B cell frequencies among the recipient mice, which partially reflect the values incurred in IgG donors. The variations in estrogens, particularly increased E2 after IgG transfer, could be linked to higher aromatase enzyme activity following a temporary peak inflammatory state. Indeed, inflammatory mediators, such as IL-6 and TNF-α, have an important role in regulating estrogen synthesis in peripheral tissues and have been shown to increase aromatase enzyme activity (599). Another reason could be the hypothetical dysregulation of the hypothalamic-pituitary-ovarian (HPO) axis, as other studies have suggested a potential autoantibody target in the second extracellular loop (ECL2) of GnRHR (546,600). While we did not assess the levels of circulating LH or FSH, an increase in LH/FSH ratio would stimulate ovarian theca cells to produce more androgens, which in turn would be converted to estradiol in the granulosa cells of recipient mice. We did note that-IgG transfer increased the frequencies of expanded DN memory B cells in blood, while ovaries and VAT had higher proportions of

unswitched memory cells and switched memory B cells, respectively. From these results we could initially speculate a mechanism for B cells in the PCOS inflammatory system based on tissue-specific shift in cellular functionality and the effects of PCOS-derived IgG, which may lead to altered energy metabolism, adipocyte differentiation and increased body weight. A similar concept has already been suggested, hyperandrogenic reprogramming of B cells which have been found capable of producing exceptionally higher levels of TNF-α (475), that can be mitigated through the action of metformin. IgG receiving mice however, did not respond negatively to other metabolic tests, which could be in line with the notion that the hormonal environment in PCOS through AR activation rather increases susceptibility to secondary triggers of inflammation, and the preceding sterile inflammatory stimuli create the conditions which allow for future metabolic dysregulation. This was first described by Winer et al. (438) as trained immunity, a form of pre-conditioning which exposed multiple target autoantigens.

As a next step, we decided to systematically phenotype B cell frequencies in DHT exposed mice which to our knowledge has never been done before. The peripubertal DHT-induced PCOS-like mouse model presented a unique grouping of B cells and circulating immunoglobulins, which only in part resembled expansions in human blood tissue. Surprisingly, we did not observe the expected increase of serum IgM. This may be due to the implications of androgen exposure at an early stage rather than peripubertal, in B cell ontogeny and its influence on PCOS inflammation. It is in fact mainly within the bone marrow during the very initial stages of development that B cells are directly sensitive to androgen receptor activation, as both pro- and pre-B cells express the AR, along with hematopoietic stem cells (HSC) (402). Unique innate-like lymphocytes that develop exclusively during fetal and early postnatal stages, remain long-lived into adulthood as their production in the bone marrow ceases (601–604). Their innate-like behaviour allows these cells to remain semi-invariant, and with a wider antibody affinity for the purpose of primary defence response (601). This is the case for a cluster of marginal zone (MZ) B cells and the B-1a subset. While the exact phenotypical description of human B-1a cells remains in question, they represent such a limited fraction in the lymphoid tissue and yet are major producers of natural IgM antibodies (NAbs), which are released at birth without prior contact with any antigen.

Potential prenatal androgen exposure leading to chronic pressure on these cell subsets may be responsible for variations of IgM titres in adulthood. A major percentage of circulating IgM is of NAb provenance playing a fundamental role in homeostatic housekeeping function towards multiple inflammatory reactions (458). While this may be purely speculative, we suggest an immune reaction towards the disease or the result of homeostatic response to an ongoing proinflammatory state driving the production of Nab – IgMs. While there is no evidence yet for PCOS, research based on lupus-prone (MRL/lpr) mice suggested that alterations of IgM titres are associated with disease severity (605).

Another potentially affected mechanism is the indirect AR regulation of B cell lympopoyesis during a more mature stage of development (606). The majority of testosterone studies focusing on the effects of B cells have been conducted on male mice or with male human data (607). Nonetheless, these studies demonstrated a general process for testosterone-dependent indirect regulation of B cell frequencies by stimulating sympathetic nervous action on BAFF-producing fibroblastic reticular cells (FRCs) (444). BAFF concentration is a dynamic steady-state which is inversely associated with peripheral B cell numbers (608,609), as well as the expression of BAFF-binding receptors. Because PCOS is such a specific hormonal environment with higher values of testosterone levels but also increased estrogen-to-progesterone ratios and high levels of circulating BAFF (442), it is plausible that the increase in BAFF concentrations cannot compensate defects in B cell development and function (527). A varying expression of the complement receptor type 2 (CR2/CD21) appeared in multiple tissues, which could be due to excessive circulating BAFF, affecting transitional B-cell maturation. Up-regulation of CD21 is directly dependent on BAFF expression and can lead to increased proliferation and Ig secretion potential (610).

AR activation is undoubtedly central to the systemic effects and B cell variations we noticed in the different tissues, as treatment with flutamide, prevented or reduced the total effect of disease. Indeed, Flutamide blocks AR signalling by preventing DHT from binding to AR, but it doesn't eliminate AR entirely. This means that residual or partial AR signalling may still occur, particularly if other mechanisms bypass Flutamide's antagonistic effects. As utilised by many studies discussed throughout this dissertation, introducing AR KO models would have instead completely eliminated AR activity,

helping clarify if the immune activation and B cell changes observed are exclusively dependent on AR, further allowing the study of differential effects also in tissue-specific knockouts. A limitation of this study may be in fact be the unresolved understanding of the cell-specific roles of androgen signalling in PCOS, which are masked in a DHT/Flutamide model where AR is present in all tissues. Another potential use of AR KO model, especially with the possibility of temporal control (e.g., tamoxifen-inducible AR KO), would be distinguishing effects of androgen receptor signalling over time. The effects of AR deletion at different developmental stages or time points could have been observed, providing insight into whether certain aspects of immune activation in PCOS require ongoing androgen receptor activity or are “programmed” early, and at what stage in immune cell ontogeny do inflammatory properties arise. This being said, each model presents its limitations, which would need to considered. In the case of an AR KO model within our study, potential compensatory mechanisms within the endocrine and immune systems that could obscure or alter the interpretation of the findings. For instance, loss of AR might lead to upregulation of other steroid hormone receptors or signalling pathways that wouldn’t be evident with DHT and Flutamide.

In our study we did not observe any metabolic nor reproductive parameters being affected by the transfer of DHT exposed B cells into reconstituted muMt^{-} mice nor in IgG-recipient T cell-deficient RAG1 KO$^{-/-}$ mice. These findings suggest that while B cells are certainly part of the inflammatory process, the exposure of B cells alone to hyperandrogenic environment is not sufficient to develop a PCOS-like phenotype.

The complete loss of mature B cells has shown to have immediate effects on glucose homoeostasis. In fact the use of Rituximab, an anti-human CD20 mAb regularly prescribed for the treatment of B cell malignancies can induce a severely impaired glucose regulation (611). It’s use in in early atherosclerotic disease, proved also beneficial to an already dysregulated glucose metabolism (612). It is worth noting that therapies based on CD20 mAb depletion strongly reduce T cell activation. Although the focus of this thesis was specifically on the alterations among the B cell lineage, the excessive levels of circulating androgens are most likely to affect also other immune cells, seeing the heterogeneous nature of PCOS pathology. In our case, the lack of B cells in muMt^{-} mice exposed to DHT, did not have a protective effect from developing both the reproductive and metabolic conditions that characterize PCO syndrome. This

result combined with the absence of any inflammation in IgG recipient RAG1 KO$^{-/-}$ mice, point towards the absolute need to further assess how other immune cells, namely T cell subsets and proinflammatory macrophages, are involved in PCOS abnormalities and chronic inflammation.

Conclusion

In summary, this thesis presents new insights into an overlooked immune regulatory mechanism in PCOS through AR signalling, which influences B cell release of harmful IgG antibodies which impact energy metabolism. Furthermore, our findings suggest caution in solely targeting CD19+ B cells as a therapeutic approach for PCOS, as our results do not fully support the idea that the absence of B cells offers protection from developing PCOS-like characteristics. The elevated IgM titres may be due to a dual housekeeping function. These results indicate that the regulation of innate and adaptive immunity within the unique hormonal environment of women with PCOS may be distinct and requires careful consideration to better understand the heightened inflammation.

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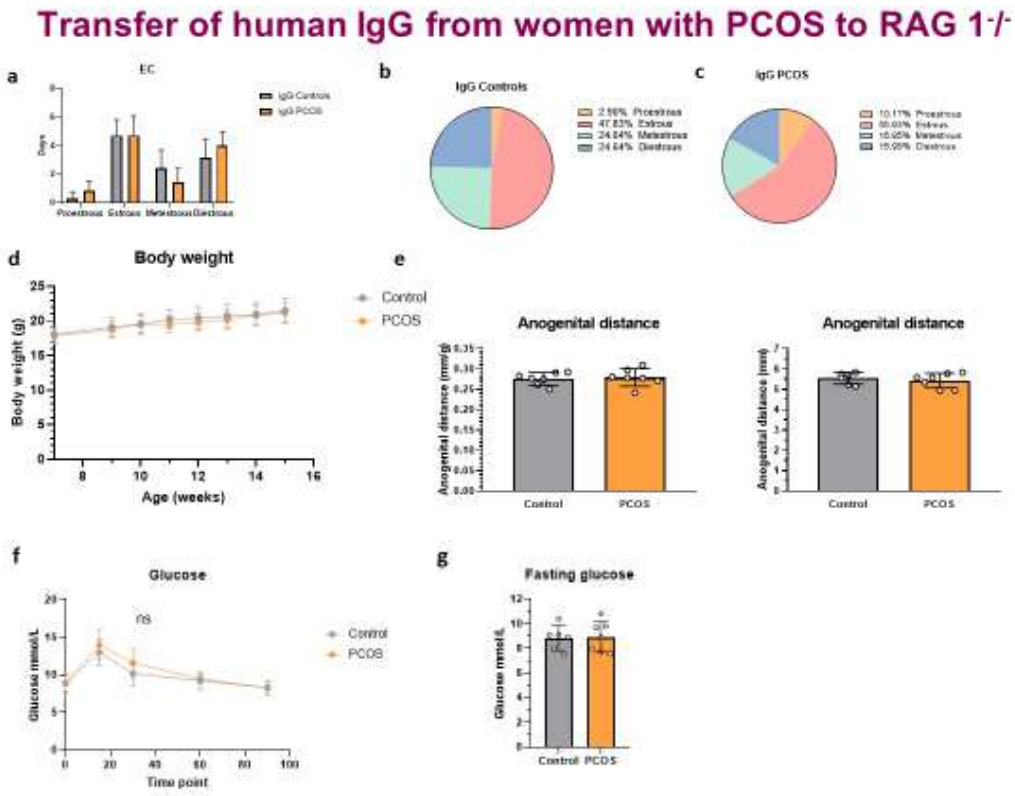
6. Appendix

Appendix Table 1. Clinical characteristics of women with Polycystic Ovary Syndrome (PCOS) and women without the syndrome.

	Controls (n=25)	**PCOS (n=25)**	***P* value**
Age (years)	36.3 (21-50)	26.4 (24-38)	**0.003**
Anthropometry			
BMI (kg/m2)	22.2 (18.3-32.2)	24.4 (19.2-39.89)	0.078
Waist-to-Hip-Ratio	0.8 (0.74-0.91)	0.82 (0.71-0.92)	0.387
Endocrine measure			
Free Testosterone (ng/mL)	0.25 (0,06-0.49)	0.43 (0.02-0.88)	**0,015**
Total Testosterone (ng/mL)	0.86 (0.8-1.74)	1.49 (0.07-3.05)	**0.019**
Free Androgen Index (FAI)	1.2 (0.22-2.48)	2.6 (0.11-20.34)	**0.021**
Androstenedione (ng/mL)	2.8 (1.17-5.99)	3.9 (2.27-6.53)	**0.002**
Metabolic measures			
Cholesterol (mg/dL)	177 (128-217)	180 (135-214)	0.597
HDL (mg/dL)	67 (46-87)	51 (36-90)	**0.001**
LDL (mg/dL)	97.3 (45.8-115.8)	108.6 (78.6-160.4)	**0.049**
Triglycerides (mg/dL)	65.5 (44-97)	82 (56-124)	**0.008**
Glucose (mg/dL)	88 (76-104)	91 (77-111)	0.452

Appendix Table 2 . Clinical characteristics of IgG donors, women with Polycystic Ovary Syndrome (PCOS) and women without the syndrome.

	Controls (n=4)	**PCOS (n=4)**	***P* value**
Age (years)	26.75 (22-31)	27 (23-35)	0.943
Anthropometry			
BMI (kg/m2)	25.19 (19.4-29.8)	24.87 (21.3-28.2)	0.909
Waist-to-Hip-Ratio	0.84 (0.79-0.91)	0.80 (0.70-0.91)	0.515
Endocrine measure			
LH (mU/mL)	7.47 (4.40 – 11.40)	15.97 (5.19-38.60)	0.351
FSH (mU/mL)	4.51 (2.74 – 6.91)	7.24 (5.59 – 8.61)	**0.058**
Progesterone (ng/mL)	8.46 (0.20 – 13.60)	0.76 (0.60 – 1.05)	0.075
Free Testosterone (ng/mL)	2.02 (0.84 – 2.68)	2.08 (0.29 – 3.03)	0.939
Total Testosterone (ng/mL)	0.27 (0.17 – 0.40)	0.39 (0.30 – 0.40)	0.107
Androstenedione (ng/mL)	3 (1.21 – 4.56)	3.67 (1.98 – 4.69)	0.516
SHBG (nmol/L)	67.08 (52.8 – 88.8)	68.05 (43.9 – 105)	0.952
Free Androgen Index (FAI)	0.41 (0.3 -0.6)	0.60 (0.5 – 0.7)	0.107
AMH (ng/mL)	3.61 (2.40 – 4.66)	7.73 (4.97 – 9.96)	**0.011**
Metabolic measures			
Cholesterol (mg/dL)	159 (132 – 184)	155.75 (146 – 172)	0.802
HDL (mg/dL)	69 (42 – 80)	60.75 (49 – 71)	0.458
LDL (mg/dL)	76 (66 – 89)	80.50 (67 – 107)	0.691
Triglycerides (mg/dL)	69 (43 – 91)	74.50 (68 – 82)	0.645



Supplementary Figure 1 : Transfer of human IgG from women with PCOS to RAG1$^{-/-}$