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Sex differences in immune response and sex hormone receptor expression in healthy individuals and during viral infection

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To my beloved wife

Lenin found music depressing.
Stalin thought he understood and appreciated music.
Khrushchev despised music.
Which is the worst for a composer?

" The Noise of Time", Julian Barnes

1 Table of Contents

2 Abstract

There is sex-bias in morbidity and mortality from infectious diseases. Infections kill more men than women and several studies have pointed out differences in the immune system as a reason. The sex hormones estrogen, progesterone and testosterone all shape the effect of the immune response on multiple levels. Women at fertile age have been suggested to have higher proinflammatory responses from inflammatory stimuli compared to men and post-menopausal women, which has been ascribed to their higher estrogen levels. This could possibly lead to a more active pathogen response but may also result in a detrimental immunopathology to infections or development of autoimmune reaction.

The overall aim of this thesis is to study the contribution of sex hormones and sex hormone receptors (SHR) to sex differences in immune response. We focus on peripheral blood mononuclear cells (PBMCs) to study such relationships in healthy individuals, as well as in individuals with asymptomatic Torque Teno Virus infection, and individuals with acute Puumala virus infection.

In **Paper I**, we investigated expression of SHR and immune response genes in PBMC from healthy premenopausal (pre-MP) women during the menstrual cycle. The expression levels were estimated using a qPCR Array (Taqman low-density array, TLDA). SHR expression did not change significantly during the menstrual cycle, but several key immune regulatory genes were significantly more expressed during the ovulatory and mid luteal phase. Further, we separated PBMC into cell subsets ($CD4^+$ T-cells, $CD8^+$ T-cells, $CD56^+$ NK-cells, $CD14^+$ monocytes and $CD19^+$ B-cells) and analyzed the expression through qPCR of estrogen receptors (ERs), ERα, ERβ1 (wildtype) and the isoform ERβ2. For the first time and unexpectedly, we demonstrate that the isoform ERβ2 was more abundant than wildtype ERβ1. The data from this paper provides new knowledge on the contribution of the menstrual cycle on immune response.

In **Paper II**, we explored the use of Torque Teno Virus as a secondary functional immune marker in men and women. Expression of viral TTV DNA in PBMCs was estimated using a qPCR kit from Argene (R-gene) and analyzed in relation to serum sex hormone levels. The results showed that 50% of the men, 25% the post-MP women, and 18% of the pre-MP women were TTV^+. Interestingly, all pre-MP women that were TTV^+ had hormonal aberrances and were either anovulatory and/or hypothyroid. TTV^+ pre-MP women also had significantly lower progesterone levels than TTV^- pre-MP women. This paper indicates that the prevalence of TTV in PBMC differs between men, pre-MP and

post-MP women. Furthermore, hormonal aberances (at least in pre-MP women) will lead to increased prevalence of TTV.

In **Paper III** we investigated the expression of ERα, ERβ1 and ERβ2 in PBMC from patients with Nephropathia epidemica, the viral zoonotic disease caused by Puumala virus, a Hanta virus known to affect more men than women. Expression of ERs in PBMCs and clinical laboratory results during the acute and convalescent phases were analyzed using a principal component analysis (PCA). The results show differences in ER expression and support previous findings that men and women have a different clinical picture

In conclusion, the results in this thesis reveal distinct patterns of immune response related to sex hormone levels, SHR expression and the phases of the menstrual cycle supporting that there a link between sex hormone levels and immune responses. Further, we show that the ER isoform ERβ2 is more abundant in PBMCs than what was previously described. The data in this thesis adds to the knowledge to the sex differences in immune response and exemplifies the importance of taking these differences into account in the clinic.

3 Abbreviations

18S	18S small ribosomal unit
ACE2	Angiotensin-converting enzyme 2
AF	Activation function
AR	Androgen receptor
ARDS	Acute respiratory distress syndrome
BERKO	ERb knock-out mouse model
cAMP	cyclic-adenosine monophosphate
CD	Cluster of diffferentiation
COPD	Chronic obstructive pulmonary disease
DBD	DNA-binding domain
DCs	Dendritic cells
DHT	5α-Dihydrotestosterone
E1	Estrone
E2	Estradiol
E3	Estriol
ER	Estrogen receptor
ERE	Estrogen response elements
ERKO	ERa knock out mouse model
FACS	Fluoresence-activated cell sorting
FDA	Food and Drug Administraton
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA3,	GATA-binding protein 3
GOI	Gene of interest
HFRS	Hanta fever with renal syndrome
HPRT-1	Hypoxanthine phosphoribosyltransferase 1
ID	Infectious disease
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
KS	Klinefelter syndrome
LBD	Ligand-bindning domain
LH	Luteinizing hormone

LTA,	Lymphotoxin-a = TNF-b
LTBI	Latent tuberculosis infection
NE	Nephropathia epidemica
NEMO	NFkB essential modulator
NFkB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
NK-cells	Natural killer cells
NO	Nitric oxide
NR	Nuclear receptor
P4	Progesterone
PAMPs	pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCA	Principal component analysis
PD-CD1	Programmed cell death-protein 1
PGR	Progesterone receptor
PUUV	Puumala virus
qPCR	Quantitative Ppolymerase chain reaction
RA	Rheumatoid Arthritis
RBCs	Red blood cells
SARS-COV2	Severe acyte respiratory syndrome coronavirus 2
SHBG	Sex hormnoe binding globulin
SHR	Sex hormone receptor
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
STI	Sexually-transmitted infections
T	Testosterone
TBX21	T-box transcription factor 21,
TGF-β	Transforming growth factor-β
TLDA	TaqMan low-density array
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TTV	Torque teno virus
UTI	Urinary tract infection
XCI	X chromosome inactivation

4 Original papers

This thesis is based on the following papers, which will be referred to in the text by paper I, II and III, respectively.

I. Peik M. A. Brundin, Britt-Marie Landgren, Peter Fjällström, Jan-Åke Gustafsson, Anders F. Johansson, Ivan Nalvarte
Expression of sex hormone receptor and immune response genes in peripheral blood mononuclear cells during the menstrual cycle
(Manuscript)

II. Peik M. A. Brundin, Britt-Marie Landgren, Peter Fjällström, Anders F. Johansson, Ivan Nalvarte.
Blood hormones and torque teno virus in peripheral blood mononuclear cells
Heliyon 6 (2020) e05535

III. Peik Brundin, Chunyan Zhao, Karin Dahlman-Wright, Clas Ahlm, Birgitta Evengård.
Gene expression of estrogen receptors in PBMC from patients with Puumala virus infection
Shock 2012 Apr;37(4):355-359

5 Enkel sammanfattning på svenska

Könsskillnader kan i många fall ses i klinisk bild och utfall av infektionssjukdomar. Infektioner dödar fler män än kvinnor och flertalet studier har visat på skillnader i immunförsvaret som en av de bakomliggande anledningarna. Könshormonerna östrogen, progesteron och testosteron påverkar immunförsvaret på flera nivåer, både gällande det medfödda och det adaptiva immunförsvaret. Hos kvinnor i fertil ålder förekommer ett kraftigare proinflammatoriskt immunpåslag jämfört med män och postmenopausala (post-MP) kvinnor, vilket antas bero på högre östrogennivåer. En kraftigare immunaktivering medger en snabbare eliminering av smittämnet, men kan vid vissa infektioner även resultera i patologiska immunmedierade reaktioner samt utveckling av autoimmun sjukdom, där kroppen angriper den egna vävnaden.

Syftet med denna avhandling är att studera hur könshormoner och könshormonreceptorer (SHR) bidrar till könsskillnaderna i immunrespons. För att dessa undersökningar har vi använt oss av cirkulerande mononukleära vita blodkroppar (PBMC), vilket omfattar B- och T-lymfocyter, NK-celler och monocyter. PBMC från friska försökspersoner, patienter med asymtomatisk Torque teno virus-infektion (TTV) och patienter med akut Puumalavirusinfektion (sorkfeber) har samlats in och analyserats.

Genuttryck uppskattades med qPCR (quantitative Polymerase Chain Reaction), en laborativ metod som används för att analysera mängden nukleinsyror (DNA och RNA) i cellmaterial. Generna innehåller information om uppbyggnaden av proteiner, centrala komponenter i cellens maskineri. När en gens DNA-sekvens blir avläst i cellen skapas en kopia av budbärar-RNA (mRNA) som är ett försteg i bildandet av proteiner. På laboratoriet renas mRNA fram ur cellmaterialet och konverteras tillbaka till en DNA-sekvens, som då benämns komplementärt DNA (cDNA). cDNA kan sedan analyseras i qPCR. Mängden cDNA blir således en ögonblicksbild på hur mycket mRNA som producerats i cellen och en indikation på hur mycket protein (t.ex. SHR) som kommer att bildas. Att mäta uttrycket av flertalet gener samtidigt innebär utmaningar, särskilt om man arbetar med små mängder cellmaterial. Då kan man använda sig av s.k. qPCR array-teknik som minskar den tekniska variationen i resultatet.

I Artikel nr 1 har vi undersökt genuttrycket av SHR och immunrelaterade markörer i PBMC från friska premenopausala (pre-MP) kvinnor under menstruationscykeln, då nivåerna av könshormoner fluktuerar naturligt. Vi fann att genuttrycket av SHR inte varierade i PBMC när vi använde oss av qPCR-array

(Taqman Low Density Array, TLDA). Däremot skilde sig uttrycket av flera immunrelaterade gener mellan olika faser av menstruationscykeln.

Från PBMC (omfattande både män, pre-MP och post-MP kvinnor) separerades sedan T-hjälparceller, cytotoxiska T-celler (”T-mördarceller”), NK-celler, monocyter och B-celler. I var och en av dessa celltyper analyserades sedan uttrycket av östrogenreceptor (ER)α, ERβ1 (den normala ”vildtypen”) och isoformen (eller en ”proteinvariant”) ERβ2. För första gången beskrivs här att ERβ2 förkommer i större utsträckning än ERβ1 hos friska individer. Resultaten från denna artikel ger ny insikt i genom vilka receptorer östrogen verkar på immunceller och hur de fysiologiska skillnaderna under menstruationscykeln bidrar till immunresponsen hos kvinnor i fertil ålder.

I Artikel nr 2 undersökte vi förekomsten av Torque teno virus (TTV) som en markör för immunfunktion. TTV är en grupp av virus som inte orsakar någon (hittills känd) sjukdom och som återfinns i blodet hos de flesta människor. TTV anses, liksom tarmfloran, vara en del av de mikroorganismer som lever i symbios med oss och därmed utgöra del vårt s.k. mikrobiom.

TTV DNA-nivåer analyserades i PBMC hos män, samt pre-MP och post-MP kvinnor. Förekomsten av TTV i PBMC var 50% hos män, 25% hos post-MP och 18% i pre-MP kvinnor. Anmärkningsvärt var att samtliga TTV^+ pre-MP kvinnor hade hormonella avvikelser (frånvaro av ägglossning och/eller brist på sköldkörtelhormon). Bland pre-MP kvinnor var också progesteronnivåerna hos TTV+ signifikant lägre än hos de TTV^-.

Resultaten i denna artikel indikerar att TTV-förekomsten i PBMC skiljer sig åt mellan män, samt pre-MP och post-MP kvinnor. Därtill var hormonavvikelser (åtminstone hos pre-MP kvinnor) relaterade till ökad förekomst av TTV.

I artikel nummer 3 undersökte vi genuttrycket av östrogenreceptorerna ERα, ERβ1 och ERβ2 i PBMC från patienter med Puumalavirusinfektion (sorkfeber), vilket drabbar män i större utsträckning än kvinnor. Uttrycket av östrogenreceptorer i PBMC och biokemiska markörer i blodet undersöktes hos patienter i akut och konvalescentfas (ca 12 veckor efter insjuknande) med hjälp av multivariat statistisk analys för att analysera multipla parametrar, s.k. principalkomponentanalys (PCA). Resultaten från PCA indikerar att män och kvinnor har olika ER-uttryck och att den sammanlagda biokemiska profilen

skiljer sig mellan könen. Dessa resultat stärker uppfattningen att det finns en könsskillnad i den kliniska presentationen av sorkfeber.

Sammanfattningsvis visar resultaten i denna avhandling att det finns distinkta mönster i vår immunrespons som kan relateras till könshormonnivåer, till uttrycket av könshormonreceptorer och till menstruationscykelns olika faser. Dessa resultat stärker tidigare rapporter att könshormoner kan påverka immunförsvaret. Här presenteras också för första gången att östrogenreceptor-varianten ERβ2 förekommer i större utsträckning än vad som tidigare rapporterats. Resultaten i denna avhandling sällar sig till de fynd som tidigare vederlagts kring könsskillnader i immunrespons och visar genom kliniska data vikten av att överföra denna kunskap till klinisk praxis.

6 Foreword

This thesis work started with discussions on sex differences in infectious diseases in Umeå, Sweden in 2009. At the time, sex differences in infectious diseases were not widely recognized and separation of data according to sex was no common practice (and there is still a long way to go). For me likewise, this was untrodden ground, and I soon realized the vast scope of this field. Getting more into the biology behind sex differences, I was intrigued by the evolutionary perspective – Why are there sex differences to begin with? And why is the female immune response to pathogens superior over male in most species from fruit flies to humans? In this thesis, apart from answering the research questions as mentioned in the Aims, I will broach the evolutionary perspective as well.

With a wide panorama of infectious diseases that clearly differ between males and females, it is surprising that the sex of patients, research animals or cell cultures are still not notified in many research reports. National institutes of Health (NIH) and Food and Drug administration (FDA) as well as the Swedish Research Council (Vetenskapsrådet, VR) have promoted the inclusion of women in clinical studies and to differentiate results by sex. However, a surprising leap forward in this field has been noted during the last year. As epidemiological data on COVID-19 revealed evident sex bias, scholars from various areas of (bio-)medical research published articles commenting and hypothesizing on this finding. This has created a general awareness and a curiosity on sex bias, and stimulated efforts to drive this research further.

A better understanding of why men and women respond differently to infectious diseases, may give more clues in fathoming the general regulatory mechanisms of the immune system, and ultimately to a different and individualized approach in treatment regimens to the benefit of all patients. My hope is that this thesis will contribute to the combined knowledge and further stimulate this development

7 Layout of thesis

Fig 1. Layout of the thesis divided into focus areas, the research questions and the papers in which they are addressed, and the respective methods used. M & M = Methods and material.

8 Background - Sex differences in immunity

Sex differences in immune response includes the response on conditions involving inflammation and thus encompass a vast group of diseases. Apart from infectious diseases, vaccination response and autoimmune diseases, other important conditions with an inflammatory component are e.g., cardiovascular diseases, thrombotic diseases, cancer and trauma-haemorrhage. In the introduction of this thesis, I will focus on sex bias in infectious diseases and have made a selection of (1) major infectious diseases and (2) conditions where sex bias is particularly prominent. I will also describe how hormonal changes will influence the immune response, on the basis of the menstrual cycle, and postmenopausal hormonal decline. During pregnancy, the female body undergoes tremendous physiological adaptation of several organ systems including the immune system. Describing the immune modulation in the pregnant woman thoroughly is beyond the scope of this thesis, and I will limit the text to the consequences the immune changes may have regarding a selection of infectious diseases, including COVID-19.

8.1 Epidemiological data indicating sex differences in infectious diseases.

Epidemiological data suggest that men and women differ in morbidity and mortality from infectious diseases as shown by Owens (2002) on data from a North American population [1]. The results from the same study indicate that the gender gap is apparent after puberty and narrowing after female menopause, indicating a possible relationship to sex hormones.

Investigating 0.5 million cases of 10 major pathogens in Brazil, and the incidence rate ratios between males and females of different age classes, Guerra-Silveira & Abad-Franch (2013) [2] established that physiological factors (including, among other factors, sex hormones and genetic differences) played a larger role to explain sex bias in incidence of infectious diseases than behavioral patterns.

In fact, there is a growing body of evidence that the immune system in males and females from various species respond differently to challenge from pathogens, in most cases to the benefit of females (reviewed in e.g. [3]) (Table 1, sex differences in immune responses). The sex bias in immune response appears on several levels, including both the innate and the adaptive immune system, and both humoral and cellular response [4]. Physiological reasons for sex bias in immune

response to infection may include differences in anatomy and metabolism, sex hormone levels, sex chromosome complement (XX and XY) – and possibly how the microbiome* [5] is constituted (Fig 2). Obviously, socio-cultural aspects including behavior factors also affect the risk of contracting infectious diseases. All of these aspects will be discussed further below.

Table 1. An overview of sex differences in immune response in various animal species (Adapted from Klein & Flanagan [3]).

Common name	Species	Immune component	Sex difference
Sea urchin	*Paracentrotus lividus*	Number of immunocytes, cytotoxic activity, phagocytolysis and haemolysis	Greater in females than in males
Fruit fly	*Drosophila melanogaster*	Activation of Toll and immune deficiency signaling	Greater in females than in males
Scorpion fly	*Panorpa vulgaris*	Haemolysis and phagocytosis	Greater in females than in males
Wall lizard	*Podarcis muralis*	Macrophage phagocytosis	Greater in females than in males
Eurasian kestrels	*Falco tinnunculus*	Hypersensitivity responses	Greater in females than in males
Great tit	*Parus major*	Hypersensitivity responses	Greater in females than in males
House mouse	*Mus musculus*	Proinflammatory cytokine responses, T-cell proliferation and antibody responses	Greater in females than in males
Rhesus macaque	*Macaca mulatta*	Proinflammatory cytokine responses and antibody responses	Greater in females than in males
Human	*Homo sapiens*	Type I interferon activity, T-cell numbers and antibody responses	Greater in females than in males

* The microbiome probably contributes more than previously expected with its vast addition of possible gene products: "*If humans are thought of as a composite of microbial and human cells, the human genetic landscape as an aggregate of the genes in the human genome and the microbiome, and human metabolic features as a blend of human and microbial traits, then the picture that emerges is one of a human 'supraorganism'*." (Turnbaugh *et al.*, 2007)

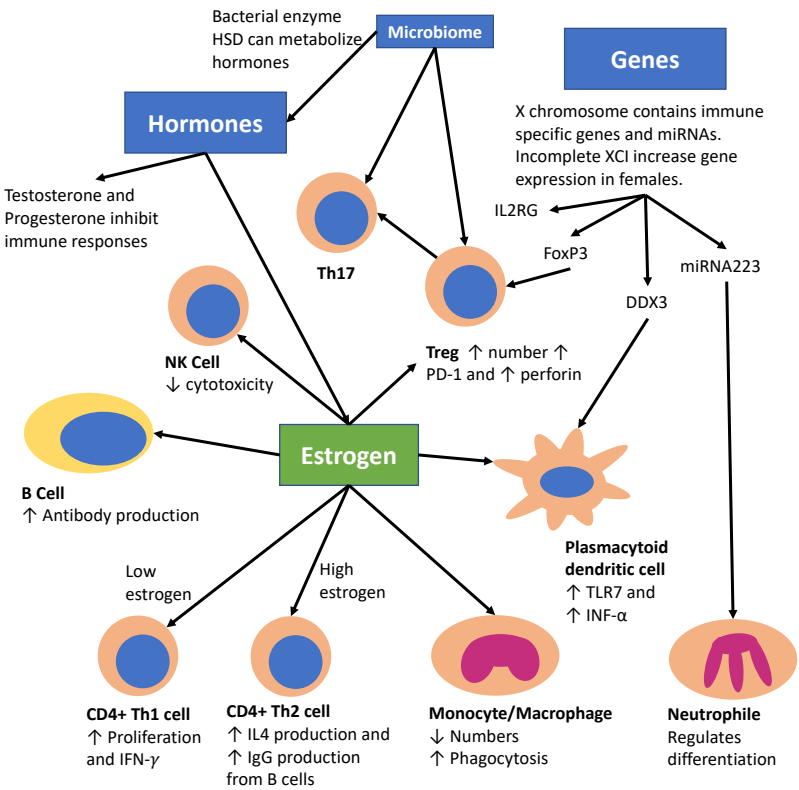

Figure 2. Sex hormones, and the genes (sex chromosome complement) may all affect the immune response. (Adapted from Klein & Flanagan [3]). Picture credit to Theo Bodin.

8.2 Infectious diseases of specific interest to this thesis

In this section aspects of immune response in important infectious diseases, and infectous diseases where sex bias is of particular interest will be briefly introduced.

8.2.1 *Hemorrhagic fever with renal syndrome*

Hantaviruses (*Bunyaviridae*) exist in at least 25 different geographical variants [6]. Hemorrhagic fever with renal syndrome (HFRS), is caused by Puumala virus (PUUV), the hantavirus that exists in Scandinavia, Russia and Northern continental Europe. This form of HFRS is also known as Nephropathia epidemica (NE). Other geographical variants of Hantaviruses are Sin Nombre virus (SNV, North America), Seoul virus (SEOV, Worldwide), Hantaan virus (HTNV, China, South Korea, Russia) and Andes virus (Argentina and Chile) [6]. Of New World Hantaviruses, pulmonary engagement is the most conspicuous clinical finding in the disease called Hantavirus pulmonary syndrome (HPS) [6]. Most hantavirus cases are reported from China, whereas Finland and Russia have the highest numbers of reported cases in Europe [7]. In Sweden, the Northern regions experience the majority of the NE cases and recurrent epidemics occur every three to four years, with occasional cases noted in the central and southern parts [8,9].

All Hantaviruses are zoonoses and PUUV is spread by the bank vole (*Myodes glareolus*, previously known as *Clethrionomys glareolus*) [6]. Human transmission is through inhaling the virus excreted through vole urine. No proven human to human transmission has been reported for PUUV, although this have been reported for other Hantaviruses (e.g., Andes virus, ANDV) [6].

NE is characterized by renal engagement; signs of acute renal failure with proteinuria are hallmark laboratory characteristics together with thrombocytopenia. Typical symptoms include acute onset of fever, abdominal and/or backpain and/or headache [10]. Hemorrhagic manifestations have been noted in 10% of cases in Sweden, of which half of them considered as severe [10]. In many cases there is a typical epidemiological history, as e.g., collecting firewood in a woodshed or cleaning out a cabin from rodent debris [10].

Most cases of NE in Scandinavia are mild to moderate, but occasional need for intensive care-treatment or dialysis may occur during epidemic years. HFRS in other regions have reached mortality rates of up to 12% and 60% for HPS [6].

Men have an overall higher incidence of NE with ~2.5 times the number of clinically reported cases compared to women [7]. However, population-based serological data from Northern Sweden indicates that similar numbers of women and men have contracted the disease and therefore have detectable antibodies [8]. Accordingly, more men than women have had symptomatic NE and received a clinical diagnosis, which indicates a sex bias in the clinical presentation. Klingström *et al.* (2008) demonstrated that the cytokine profile differs between male and female patients [11], however, no reports exist that clearly states any relation between symptoms and the sex of the patient.

8.2.2 ***Torque teno virus***

It is not entirely correct to state Torque Teno Virus (TTV) as an infection, rather TTV should be regarded as a commensal virus and therefore a part of our microbiome [12]. TTVs are a group of 29 species (TTV1-29) that belong to the genus *Alphatorquetenovirus* of the family *Anelloviridae* [13]. *Anelloviridae* are circular single-negative-strand DNA-viruses and are found in blood serum and lymphocytes [14]. They are almost ubiquitous in the human population (e.g., 94% in Russian population [15]) and it is possible that limitations in the detection methods used underestimates the general prevalence which may be close to 100% [14]. A study of a population in Austria further showed that the TTV-levels in blood were increasing with age and were higher in men [16]. Since the discovery of TTV in a Japanese patient 1997 [17], no connection to pathology has yet been established [18].

TTVs are found in many animal species, but the types of virus are species-specific and TTV co-evolution with their host have been suggested. It is likely that most species carry TTV [19]. As certain TTVs are specific for humans, they can be used as a marker for human presence. Human wastewater will contain human-specific TTV, and detecting TTV is therefore used a marker of human presence or inadequate sanitation measures [20].

Clinically, TTVs have been proposed to act as a secondary *functional* marker for the immune system, especially in studies on transplanted patients [21-23]. Even though apathogenic, TTV DNA-levels in serum will be controlled by the immune system. A functional immune system will contain the virus at low levels, however, if the host is immunocompromised by any reason (by pharmacological treatment, other infections, etc.) TTV levels will rise [22]. Therefore, the high levels of TTV can be considered as a marker for a compromised immune system.

The virus evidently evades clearance of the immune system, and one proposed mechanism have been through microRNAs (miRNAs) that downregulates IFN production [24]. Women have a more robust type I IFN production due to a higher

and more variable expression of *TLR7* (Toll-like receptor 7) and *IRF5*, (interferon regulatory factor 5) as discussed below (see section (8.5.4). This may explain the lower levels of TTV-DNA found in women compared to men [16].

8.2.3 ***Other infections with sex bias***

8.2.3.1 *Respiratory viral diseases including influenza and COVID-19*

Sex differences in viral respiratory diseases have particularly been studied with a focus on sex differences in the innate immune response. Influenza is one infection where pregnant women are more at risk[25]. This has been shown in clinical studies and epidemiological reports both for seasonal influenza and pandemic influenza. During the "Spanish flu" (1918-1918) there were more male casualties. The pandemic coincided with WWI, which may have contributed to a higher male exposure [25]. During the "Asian flu" (1956-1957), however more women than men died, and the latter pattern is repeated in studies on seasonal influenza during recent years [25].

Since the detection of a novel coronavirus (SARS-CoV2) in Wuhan, China in late 2019 and during the subsequent COVID-19 pandemic, additional data on sex bias in morbidity and mortality from viral respiratory infection has accumulated. Numerous reports have described that men have a higher risk of severe disease and death (female/male ratio 1:1.7) [26]. Contributing underlying factors have therefore been examined. With advanced age, the prevalence of comorbidities (e.g. cardiovascular diseases, COPD and diabetes) increase, which puts elderly at higher risk for severe disease. Smoking (including more males from an international perspective), leads to damage of the lung epithelium and in general increased vulnerability to viral air-borne infections. Active smokers and previous smokers are more at risk for hospitalization, ICU admission and death of COVID-19 related to the number of pack-years exposed to tobacco smoke [27].

Both declining levels of estrogen (women) and testosterone (both men and women) have been considered as reasons to increased inflammation and therefore a risk for severe covid-19 disease. Estrogen and testosterone have distinct and overlapping functions, and both share anti-inflammatory properties under certain conditions [28]. A decrease in testosterone is associated to COPD, diabetes and obesity [29]. These are all comorbidities that will increase the risk for the patient of severe disease course of COVID-19 [28]. In Sweden, 12% of women and 16% of men aged 65-84 have diabetes [30]. Further, a decline in testosterone leads to lower muscle strength, including respiratory muscles which could

predispose the patient to development of severe COVID-19 with development of acute respiratory distress syndrome (ARDS) [31].

Sex bias is ultimately reduced to the type of immune response elicited in men compared to women. A higher and more variable expression of TLR7 and IRF5 in women results in a more robust type I IFN (IFN-α) response. TLR7 and IRF5 are both genes encoded on the X chromosome. Genes encoded on the X chromosome are more variable in females as they have both a paternal (X_p) and maternal (X_m) copy (see section 8.5.4). The stronger inflammatory response (IL-6, IL-18 and IL1-β) seen in men could be the result of SARS-CoV2 viral evasion of the weak IFN-response in men [26]. The failure of one microbial "immune sensor" may be compensated for by others through compensatory mechanisms of other pathways (Fig. 2) [32]. This could result in an excessive onset on inflammatory cytokines leading to the detrimental clinical picture seen in severe COVID-19, more common among males [26].

Apart from immune genetic differences related to sex chromosome complement and sex specific age-related epigenetic changes (see sections 8.5.2.3.4. and 8.5.3.3.), epigenomic imprinting of immune cells at a young age may also explain sex differences that are still present in elderly patients [33].

Furthermore, sex-based differences in the expression and regulation of molecules important for viral entry into the host cell have also been proposed. Angiotensin-converting enzyme 2 (ACE2) and transmembrane protease/serine subfamily member 2 (TMPRSS2) are molecules vital for cellular entry of the SARS-CoV-2. As ACE2 is associated with IFN gene expression (which do show sex bias, as mentioned above), it is possible that the intrinsic cell regulation of ACE2 may change with age, sex steroid levels and viral challenge, which triggers production of IFN [34].

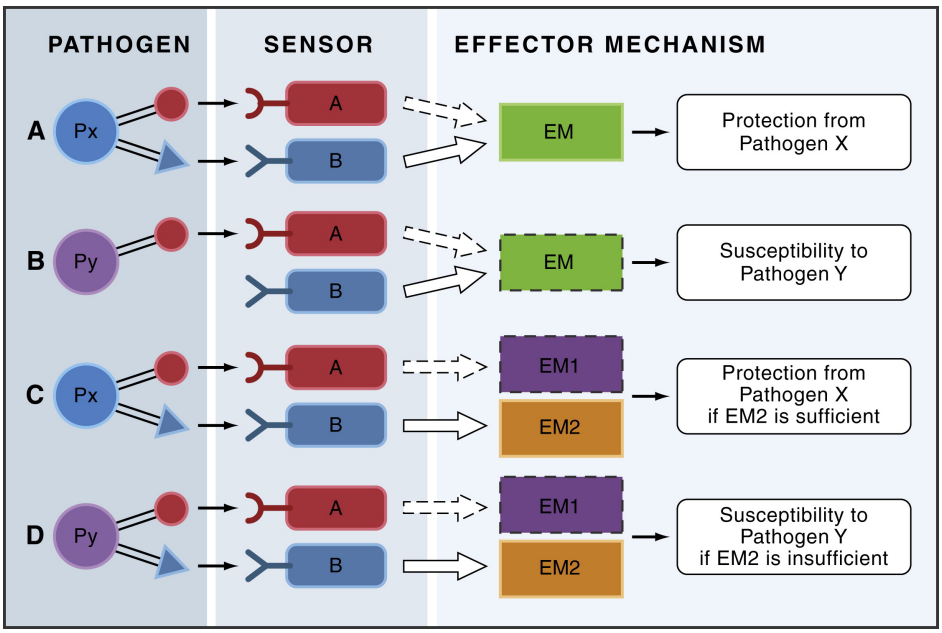

Fig 2. Pathogens Px and Py are detected by immune sensor A and/or B and activates an effector mechanism. A defect pathway A can be compensated by pathway B in the case of pathogen Px (A), but not in the case of pathogen Py (B). A defect EM1 can be compensated by EM2 if EM2 is sufficient to provide protection against pathogen Px (C). If EM2 is not sufficient to protect against Py, then EM2 will not compensate for EM1 deficiency (D). Solid lines indicate intact pathways; dashed lines indicate inactive pathways. Pathway deficiency can result from mutations or other changes in the immune system or can be due to pathogen evasion. Reprinted from Immunity 34 (5) Nish&Medzhitov, 2011 [32], Host defense pathways: role of redundancy and compensation in infectious disease phenotypes. Page .633 Copyright 2011, with permission from Elsevier.

8.2.3.1.1 Respiratory viral diseases in pregnant women

Notable is, that pregnant women represent 1% of the population at a given time, however, they represent 5% of the fatal cases of influenza [25]. This is probably both due to hormonal changes of the immune system and as a result of the cardiopulmonary changes associated with pregnancy [25]. Normally, the mother will have an increased cardiac output starting in the first trimester and a progressive increase in afterload during the pregnancy. The lung capacity and colloid osmotic pressure will be decreased. This puts pregnant women at risk for developing pulmonary edema and increase the risk for mechanical ventilation. The risk for mechanical ventilation of a pregnant influenza patient is 33% higher than compared to age-matched non-pregnant women [25].

On the basis of the increased risk for severe influenza in pregnant women, Sulentic *et al.* (2020) analyzed the current published case reports on Covid-19 as of October 2020 [35]. Initially, Chinese reports stated no increased risk for pregnant women. However, recently published case series report increased risk for morbidity and mortality in pregnant women, including preterm birth, fetal growth restriction, and maternal development of severe lung edema and sepsis [35].

8.2.3.2 *Paracoccidioidomycosis*

Paracoccidioides brasiliensis, is an endemic thermically dimorphic fungus found in Central America and tropical South America. Paracoccidioidomycosis deserves mentioning as an example of extreme sex bias. A male/female ratio of 13-70:1 has been reported in case series from South America in post pubertal patients, whereas no sex difference occurs before puberty [36]. Skin testing with paracoccidioidin antigen revealed (similar to HFRS above) that girls and boys and women and men were equally exposed to the fungus but differ in disease development [36,37]. Restrepo *et al.* [38] demonstrated through a series of *in vitro* experiments, that estrogen at physiological levels inhibits the transition from mycelia or conidia to yeast and as a consequence impedes the pathogenicity of the fungus. Apparently, E_2 binds to a fungal protein which acts as a receptor for estrogen and therefore alters the fungal gene expression [36].

8.2.3.3 *Sepsis*

There are epidemiological reports with conflicting results on sex differences in sepsis. Both an increased male and female mortality have been described [39].

Studying sepsis is in a clinical setting is challenging. Sepsis is by nature a diverse diagnose that includes bloodstream infections due to a wide selection of organisms, and its presentation depends on multiple factors (e.g. site of infection, host comorbidities and immune status), which may all influence clinical presentation, severity and outcome.

Early initiation of antibiotic treatment is crucial and have proved to be a major determinant of mortality. One study found that women experience longer delays to initial antibiotics than men, even after adjusting to infectious source [40]. And men have proved to be reluctant to seek healthcare in general [41]. Sociocultural factors as patient's and doctor's delay may therefore contribute to the perceived sex bias. Additionally, treatment protocols for sepsis with antibiotics, fluids and vasoactive catecholamines are based on studies of male participants, which therefore may increase the risk of side-effects in women or even prove less efficient [39]. Results on human studies may therefore be confounded by both gender aspects, sociocultural issues, treatment regimens and physiological factors (as sex hormones).

Animal studies allows better control of confounding factors and results from these studies demonstrate a more consistent favorable female outcome for sepsis and other models of shock (e.g., trauma-hemorrhage [T-H]). T-H and sepsis may lead to dysfunction of several organ systems, and E_2-administration provided salutary effects on both cardiac and hepatic function [42]. Improved hemodynamic stability through treatment with E_2 probably relates to upregulation of cardiac heat shock proteins (HSPs), a group of endogenous protective proteins [43]. By administering E_2 to rats following induced T-H, Hsu *et al.* (2008) showed that p38 mitogen activated protein kinase (MAPK) was involved in the inflammatory process and that E_2 could reverse the inflammation [44].

8.2.3.4 *Urinary tract infections.*

It is generally appreciated that women get more frequent urinary tract infections (UTIs) than men. Traditionally this has been attributed to anatomical differences in urethral length. The incidence for lower UTIs is less common in men >18 years old (3%) than women (12.6%) of the same age [45]. However, complicated UTIs with fever (pyelonephritis) appears more often in men [46]. Apparently, mild disease is common in women and severe in men, and the most pronounced sex difference is found among non-geriatric adults [47]. This may indicate a role of sex hormones, yet, studies have failed to elucidate their exact roles [48].

8.3 Sex differences in immune response

Numerous studies have reported sex differences related to the innate response, but the adaptive immune system also exhibits sex specific characteristics. Sex hormone receptors (SHR), which will be described in a separate section (8.5.2), are present in several immune cells which accordingly have the molecular prerequisites for interacting with sex hormones [49,50].

Apart from the presence and distribution of SHR, sex differences in immune response are also be the result of genetic and epigenetic aspects. Below, some of the most important aspects on sex differences in the immune response will be summarized.

8.3.1 ***Sex differences in the innate immune response***

As the innate immune response is initiated, signaling through chemokines and cytokines locally and globally will evoke and give time to the slower adaptive immune response to commence. Cells included in the innate response are neutrophils, monocytes/macrophages, NK-cells and dendritic cells harboring cell-bound pattern recognition molecules (PRMs, e.g. Toll-like receptors [TLRs]) that recognize pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). The humoral arm of the innate immune

system includes soluble molecules that activate the complement system and opsonization of microbes [51].

Macrophages, derived from circulating monocytes, are a major source of cytokines (e.g. IL-6 and TNF-α) of the acute phase systemic response, and in the recruitment of cells of the adaptive immune response. In mice, females have, compared to males, higher numbers of both pleural and alveolar macrophages and increased phagocytic capacity. This may be attributed to higher levels of TLRs in female mice [51]. In vitro experiments demonstrate that E_2 at pregnancy levels will decrease TNF-α production from monocytes, probably through inhibition of NFκB [52].

Dendritic cells (DCs) are critical cells in the initiation of the immune response. DCs may perform antigen capture in one location and antigen presentation in another. Plasmacytoid dendritic cells (pDCs) are important cells for pathogenesis of viral immunity and autoimmune reactions [53]. Viral nucleic acids (and self-nucleic acids) may trigger the production of type I interferons (IFN-α/β) which are potent immunostimulatory cytokines. An overproduction of IFNs is central in development of systemic lupus erythematosus, and pDCs are an important source. Seillet *et al.* (2012) showed that estrogen enhance IFN-production in pDCs through TLR7 and TLR9, and that the mechanism is regulated through intrinsic expression of ERα [53]. On the other hand, progesterone downregulates INF-α, mediated through TLR9 [54].

8.3.1.1 *NFκB and sex hormone signaling*

ER interacts with several intracellular transcription factors that influence the gene transcription. Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB) is an inducible transcription factor that controls expression of several stress response genes and NFκB is crucial in the development of innate immunity. Among NFκB target genes are regulators of inflammatory cytokines, cell survival, proliferation and cell surface proteins [55-57]. Toll-like receptors (TLRs), which are critical in the activation of the innate immune system, activate NFκB as a response to pathogen associated molecular patterns (PAMPs). The activity of NFκB may be further affected by the influence of several other factors including steroid hormone signaling.[56]

8.3.1.2 *Adaptive immune response*

The adaptive immune response is the targeted immune response tailored to react to specific pathogen triggers. The adaptive immune response is slow, as specific memory B- and T-cells need to be activated and propagate signals to promote selected cell proliferation and effector mechanisms. During the course of evolution, the adaptive response has been shaped and refined to recognize threats

from pathogens and avoid attacks on host targets. Notwithstanding, autoimmune reactions may occur if the checkpoint limiting mechanisms of autoreactive T-cells or autoantibodies from B-cells are failing. Lymphocytes are the key cells of the adaptive immune response, but their response is intertwined with signals and effects from macrophages, dendritic cells and neutrophils.

8.3.1.2.1 Estrogen influence activity and numbers of B-cells and T-cells

SHR are present in immune cells, including lymphocytes [49] and estrogens suppresses both B- and T-lymphopoesis [58]. However, E_2 stimulates B-cells to immunoglobulin (Ig) production, which leads to a higher baseline Ig- production in women than men, and a higher diversity of B-cells [59]. E_2 reduces apoptosis of immature B-cells and therefore more autoreactive B-cells may be released from central and peripheral checkpoints [60]. Development of high-affinity autoreactive antibody species is driven by E_2, which increases somatic hypermutation and class-witch recombination of B-cells. This may explain the dual effect of estrogen with both increased humoral immunity towards pathogens and autoreactivity [60]. On the contrary to the above-mentioned estrogenic effects, testosterone will inhibit class-switch recombination which could further enhance sex difference in both infectious and autoimmune diseases.

The effect of estrogen on T-cell development is complex. Involution of the thymus, were T-cells mature and undergo positive and negative selection, is promoted by E_2 [58]. Women have a higher $CD4^+$ T-cell counts and a higher $CD^+/CD8^+$ ratio, while men have higher $CD8^+$ T-cell counts [3]. The activity and distribution of $CD4^+$ T_H-cells differs between men and women. Several studies have demonstrated a biphasic effect of E_2 on T-cell polarization as low E_2-levels correlate with T_H1-cells and high levels with T_H2-cells [61,62]. Of note, the previous paradigm of dividing T_H-cells into T_H1- and T_H2-cells have been questioned as of the discovery of T_H17 and T regulatory cells (Treg) [52,63]. Tregs, a subset of $CD4^+$ T_H-cells, are important in tolerance and maintenance of autoimmunity, and dysregulation of these cells have been associated to development of autoimmunity disorders [58]. Interestingly, Arruvito *et al.* (2007) [64] noted that The numbers of Treg cells are correlated to E_2-levels through the menstrual cycle.

8.4 Sex differences in non-communicable diseases

8.4.1 *Autoimmune diseases*

Several autoimmune diseases have a clear sex bias. The overall prevalence for systemic lupus erythematous (SLE) in men vs. women is 9:1, which is further

increased during the female reproductive years. Similarly, rheumatoid arthritis (RA) is more prevalent in men by 3:1 [65].

Estrogens will exacerbate disease progress of SLE and pregnant women may experience disease flares [52]. In SLE, the type I IFN (IFN-α) inflammatory response is important for disease development. As discussed in the genetic background for sex differences (section 8.5.4), type I inflammatory response is more robust in women due to the sex chromosome complement. In addition to this, estrogen is an enhancer of type I inflammatory response [65]. The development of multiple autoantibodies, including anti-nuclear antibodies (ANA), is a distinctive feature of SLE. The development of autoantibodies and reduced apoptosis of B-cells is promoted by E_2 (see section 8.3.1.2.1), and it has been suggested that an impaired ability to process and remove dying cells is driving SLE and accounts for continued development of antinuclear antibodies [52].

In patients with RA, even though there is a distinct sex bias, the role of estrogen and progesterone is not fully established. Typically, and in contrast to SLE, pregnancy alleviates arthritic symptoms, and they may even go in full remission. However, studies on the use of hormonal replacement therapy (HRT) have failed to demonstrate a clear clinical benefit [65]. Interestingly, the number of gestations has been linked to risk of developing RA. The reasoning for this may be repeated triggering of B-cells of the semiallogenic fetus [52].

8.4.2 *The role of estrogen and ER in cancer*

The risk of malignancy is much higher in men than women for a majority of cancers at most ages [66]. Survival of cancer is generally similar in men and women, so the difference is largely in incidence [67]. Both environmental (e.g. tobacco smoke, occupational exposure and UV-light) and physiological factors contribute to these differences. Of physiological factors, sex hormones and particularly estrogens, have been identified in breast cancer [68] but also in e.g. colon cancer [69].

For this reason, ERα and ERβ have both been focus of intensive studies in oncology. ERα is present in about 10% of normal breast epithelium, but to 50-80% in breast tumors. Accordingly, ERα expression seem to promote both tumorigenesis and progression of breast cancer [68]. On the contrary, ERβ is expressed to 80% in normal breast epithelial cells, but the expression of ERβ is decreased or is lost in breast tumors. The exact role of ERβ2 is unclear, with studies showing conflicting results [68]. Similarly, ERβ expression is allayed in colonic cancer with diminishing levels or ERβ corresponding to loss of differentiation and advanced Dukes staging [68].

8.5 Reasoning behind sex differences

8.5.1 *Anatomical and physiological differences*

The mucosal epithelium is an entry site of infections. Women have a cervico-vaginal mucosal epithelium which area is larger than the mucosal epithelium of the male penis. The larger area in addition to a higher risk of damage to the epithelium during intercourse puts women more at risk of contracting sexually transmitted infections (STIs). Also epithelial thickness, frequency of Langerhans cells and presence of Lactobacilli differ between men and women and are affected by sex steroids [4]. Differences in anatomy, body composition (e.g. fat distribution), physiology (e.g., gastric emptying and renal clearance), as well as drug distribution volume and liver metabolism all possibly contribute to sex bias in morbidity and treatment effect of infectious diseases [70,71].

The human host is in a symbiotic relationship with the immense numbers of microorganisms, that constitute the microbiome [5]. Sex influences the microbiome also outside the reproductive tract, and most likely sex hormones are involved in shaping this microbiome. Perturbation of the gut microbiome may predispose for inflammatory diseases, diabetes, and infections like Clostridioides difficile enteritis [3].

8.5.2 *Hormonal differences – Sex hormones and Sex hormone receptors*

8.5.2.1 *Sex hormones and their synthesis*

Sex hormones (SH) are composed of a steroid molecule with a four-fused-ring structure and are all derivates from cholesterol. SH are grouped into estrogens, androgens and progesterone. Estrogens are produced both in gonads and extragonadal tissue. 17β-estradiol (E_2) is the most potent estrogen in men, post-MP women and non-pregnant women. In pre-MP women, E_2 is produced in the granulosa cells and thecal cells of the ovaries. The less potent estrogens estrone (E_1) and estrone (E_3) are metabolites of E_2. Extragonadal estrogen synthesis occurs in several tissues including adipose tissue, breast, osteoblasts and chondrocytes of bone [72].

Cholesterol is first transformed into progesterone (P_4), which is the precursor of cortisol and aldosterone (both produced in the adrenal glands), as well as the androgen androstenedione. Androstenedione is then be further transformed into estradiol and testosterone (T). T may be converted to E_2 by the enzyme aromatase or reduced to dihydrotestosterone (DHT) by 5-α reductase.

8.5.2.2 *Nuclear receptor family*

Sex hormone receptors (SHRs) belong the nuclear receptor (NR) family together with among other glucocorticoid receptor, retinal acid receptor, thyroid hormone receptor, vitamin D receptor and several "orphan receptors" with unknown function (ref). The SHR group comprises of ERα, ERβ, androgen receptor (AR) and progesterone receptor (PGR). As SH are lipophilic steroids, they diffuse freely through the plasma membrane and bind to the intracellular SHRs. ERs, ARs, PGRs do all have a steroid ligand that allow intracellular access.

8.5.2.3 *Estrogen receptors*

8.5.2.3.1 Structure of ERα, ERβ and their isoforms

Since the discovery of ERα (1987) [73] and ERβ (1993) [74], their structure, distribution and pathways of signaling have been extensively mapped. ERα and ERβ, although located on distinct genes, have a high degree of sequence homology and largely differ in the NH_2-terminal. The full-length receptors are 595 (ERα) and 530 (ERβ) amino acids long which encode proteins of 67 and 60 kDa respectively [75]. The structure of ERs is divided into a DNA-binding domain (DBD) and ligand-binding domain (LBD) connected by a hinge region. Activation function 1 (AF-1) and AF-2 domains act as binding sites for cofactors. The amino terminal contains the activation function 1 domain (AF-1), that mediates activation independent of a ligand, while AF-2, located in the LBD is strictly

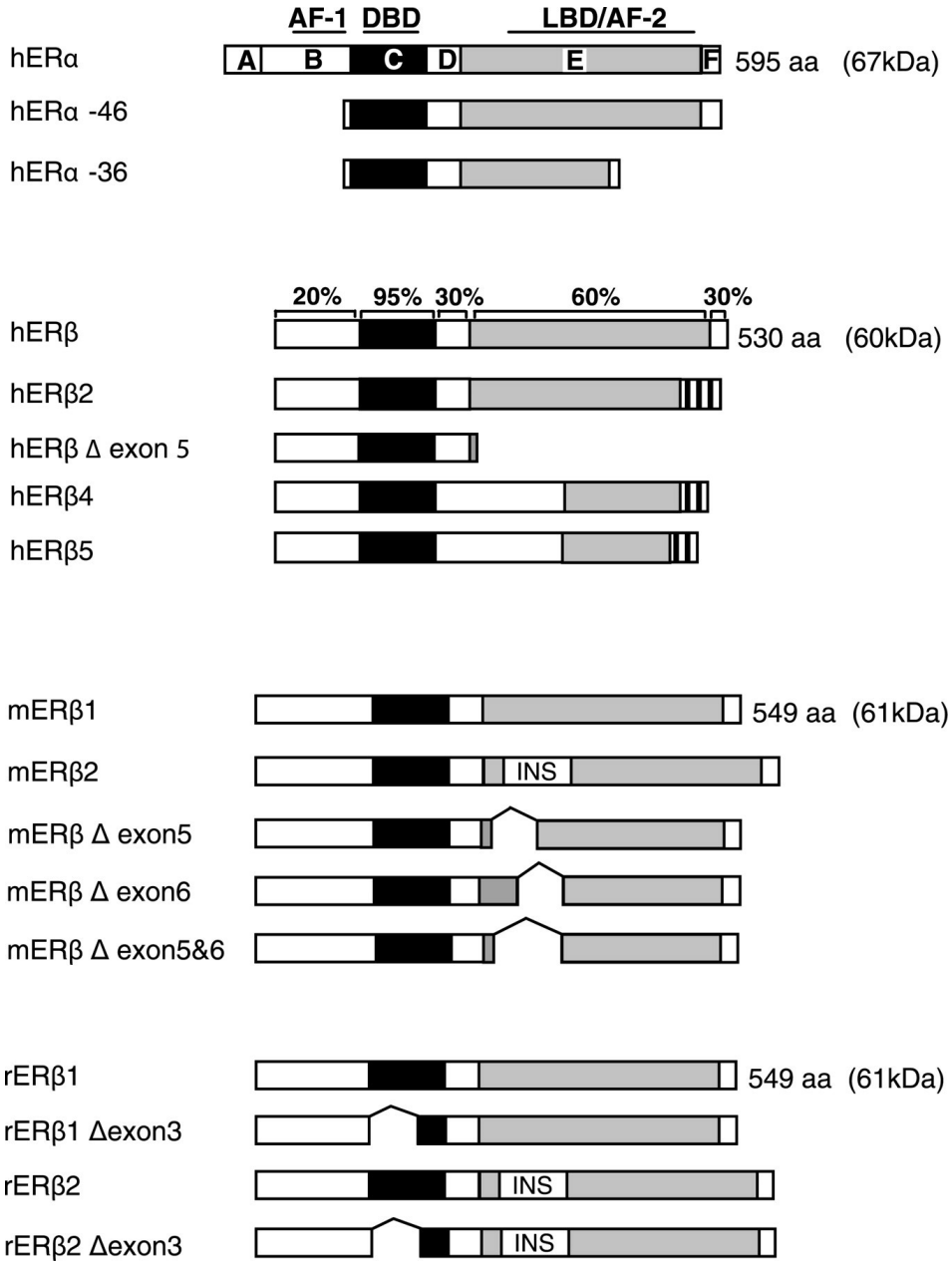

Fig. 3. Schematic overview of decribed isoforms of ERα and ERβ. Published with permission from Physiological reviews, Heldring et al.[75], Copyright 2007, The American Physiological Society.

ligand dependent (Fig. 3). The wildtype receptor transcript may be further modified into different isoforms by e.g., splicing mechanisms before translation into a protein.

8.5.2.3.2 Classical estrogen receptor signaling

Inside the cell, E_2 binds to the DNA-binding pocket of ER held in an inactive state by chaperone proteins. Upon E_2 binding the ER dissociate from the chaperons and corepressors, homodimerizes, attracts coactivator proteins (see below), and binds to specific regions on the DNA called estrogen response elements (EREs), that are typically found in the promotor or enhancer regions of E_2 target genes. ERα and ERβ have similar affinities for estradiol (E_2, see section (8.6.2.3.3) and bind to both unique and overlapping DNA regions [75] (Fig. 4).

The homodimerization (αα, ββ) is needed for ERs to reach its active state. Heterodimers, i.e. αβ, is also possible, however, the αβ heterodimer generally mediates ERα deactivation. Combinations including isoforms have also been described (e.g., $\alpha\beta_1$, $\alpha\beta_2$, $\beta_1\beta_2$), but the exact roles of these combinations have not been fully understood [76,77].

8.5.2.3.3 ER ligands and affinity

Steroid molecules have a lipophilic nature and may therefore readily diffuse through the lipid bilayer of the plasma membrane (and the blood-brain barrier). There are three endogenous estrogens which all acts as ligands: Estrone (E_1), estradiol (E_2) and estrione (E_3). E_2 is the dominant estrogen in pre-MP females an also the ligand that binds ER with the strongest affinity. The affinity of E2 to ERα and ERβ is similar. Interestingly, E_1 has a higher affinity for ERα, while E_3 has a higher affinity for ERβ. Different estrogen metabolites have also proved to have a selective affinity for ERα or ERβ [78].

8.5.2.3.4 Cofactors interacting with nuclear receptors

Cofactors (coactivators and corepressors) influence the activity of nuclear receptors (NR). A multitude of coactivators and corepressors have been described, which acts in a complex with NRs to mediate a balanced and adequate transcriptional response. p160/SRC, especially SRC-3, (Steroid receptor coactivator, also known as amplified in breast cancer-1, AIB1) and p300/CBP (cyclic AMP repressor binding-protein or CREB) have been recognized as two of

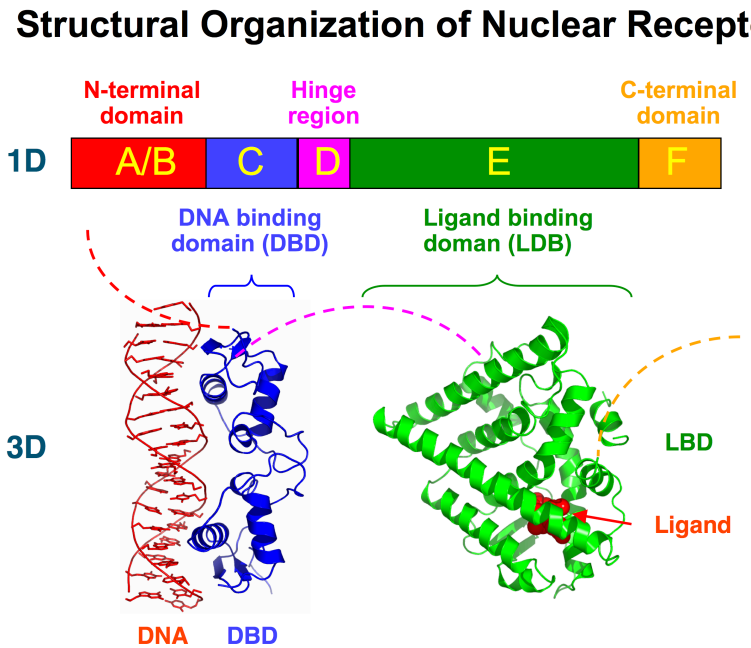

Figure 4. Structural organization of nuclear receptors. The DNA-binding domain (DBD) is the region of the receptor that binds into the DNA. The ligand binding domain (LBD) will harbour bind the ligand (steroid molecule, e.g. estradiol or testosterone). Picture credit Ivan Nalvarte.

the most important activators (Fig. 5). Activation includes chromatin remodeling, histone acetyltransferase (HAT) and RNA polymerase mediation. p160/SRC and p300/CBP are histone acetyltransferases which opens up the chromatin for transcription by loosening the DNA-histone binding through addition of acetyl residues to the respective histones [79]. Histone deacetylases (HDACs) on the other hand removes acetyl residues which inhibits gene transcription.

In the absence of a ligand, corepressors have access to the ligand-binding domain (LBD) of the NR. As the NR binds a ligand, a conformational change takes place in the LBD which release the corepressor and allows DNA binding and association with coactivators [79]. Chromatin immunoprecipitation (ChiP) and DNA deep sequencing (ChiP-seq) of whole genomes have evinced new NR binding sites challenging the traditional models of NR gene activation.

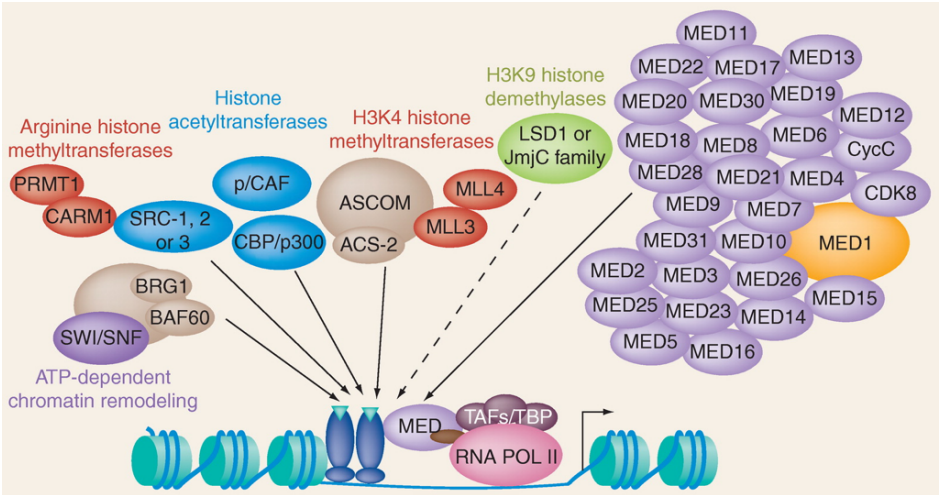

Figure 5. Histone methylation and acetylation as means of epigenetic regulation of nuclear receptor transcriptional activity. Histone acetylation opens up the chromatin for gene transcritption, deacetylation inhibits this process. Methylation. Published with permission from Epigenomics, Green & Han [79], Copyright 2011, Future medicine Ltd.

8.5.2.3.5 Membrane-initiated steroid signaling

Membrane-initiated steroid signaling (MISS) involves G-protein coupled receptors (e.g. GPER-1) which function through activation of downstream pathways including cAMP, mobilizing intracellular Ca^{+}, K^{+} currents and NOs. The role of MISS is not fully understood. However, signaling through nuclear ER that bind directly to DNA-regions usually gives effects within hours-days, while membrane ER may exert their effect within seconds-minutes [80]. Rapid effects of estrogen signaling (in e.g., cardiovascular regulation) are believed to be mediated through MISS.

8.5.3 *Fluctuation in hormone levels*

Throughout the lifespan, levels of circulating sex hormones vary in both males and females. Before onset of puberty the sex hormone levels are low, and the highest levels of estrogen and progesterone in women and testosterone in men will be reached during the reproductive years. A transient and sex-specific rise in sex hormone levels during the first year of life, known as “minipuberty” is evident and suggested to be involved in development of several organ systems, including the reproductive system, somatic growth and neurological development [33,81]. The effect of the “minipuberty” corresponds with a preponderance for male incidence of infectious disease according to epidemiological data [2].

8.5.3.1 *Menstrual cycle hormone fluctuations and the immune response*

The length of menstrual cycle varies greatly (21-35 days) in women but is usually around 28 days long and is characterized by fluctuations in serum sex hormones [82]. Following menstrual bleeding, in the beginning of the follicular phase, P_4 and E_2 levels are low. The latter starts to increase as a result of FSH-stimulation of the ovaries and E_2-levels peak close to the ovulatory phase. In the ovulatory phase LH rise sharply which triggers the release of the ovum. The following luteal phase is characterized by a quick decline in LH. E_2 declines temporarily before it's second smaller peak. If no conception has occurred, P_4 will be produced from the corpus luteum. The zenith of P_4 will be in the midluteal phase and its withdrawal elicits the endometrial changes resulting in menstruation (Fig. 6).

The balance and release of hormones is regulated through the hypothalamic-gonadal axis (HGA) where neurosecretory cells release gonadotropin-releasing hormone (GnRH) into the hypothalamic-hypophysial portal circulation. The anterior pituitary gland responds with releasing FSH and LH which control gonadal function in women (as described above). In men FSH and LH have important functions for spermatogenesis and production of androgens, respectively.

All the above-mentioned hormones have the possibility to affect numerous changes in not only the reproductive system, but as decribed in this text also in the immune response. The immunological modulation during the luteal phase, resulting in a more tolerant immune system, will allow implantation of a semi-allogenic embryo but also opens up a "window of vulnerability" for pathogens contracted especially through the female reproductive tract (Fig. 6) [82]. The immune response shift seen during the menstrual cycle is suggested to be regulated through NFκB (see section 8.3.1.1) [83].

The fluctuating levels of E_2 also alters the activity of autoimmune disorders and infectious diseases. Infectious diseases such as latent tuberculosis infection (LTBI) may e.g. be activated during pregnancy and puerperium, and autoimmune disorders are more likely to be worsened in their presentation during the follicular phase (e.g. RA) or luteal phase (e.g. SLE, fibromyalgia, multiple sclerosis).[84]

There is a dearth of clinical studies available on how menstrual hormonal changes affect infectious diseases. Benki *et al.* (2004) have described that the number of HIV-particles released in cervical secretions by HIV^+ individuals are lower close to ovulation, which coincides with the LH peak.[85] The risk of contracting HIV is also varying during the menstrual cycle, with the highest risk being during the luteal phase [86]. Hormonal changes affect both immunological modulation on a systemic level and local protective factors in the lower genital tract (mucosal fluid

viscosity, pH, and antimicrobial protein composition and epithelial barrier thickness). A low pH (as a result of lactobacillus presence) is favorable in the protection against HIV [87]. The increased sensitivity to HIV is attributed to the higher levels of progesterone in the luteal phase, which is exemplified by women on treatment with contraceptives containing synthetic progestins who have a 1.5-2-fold increase in the risk of contracting HIV [86].

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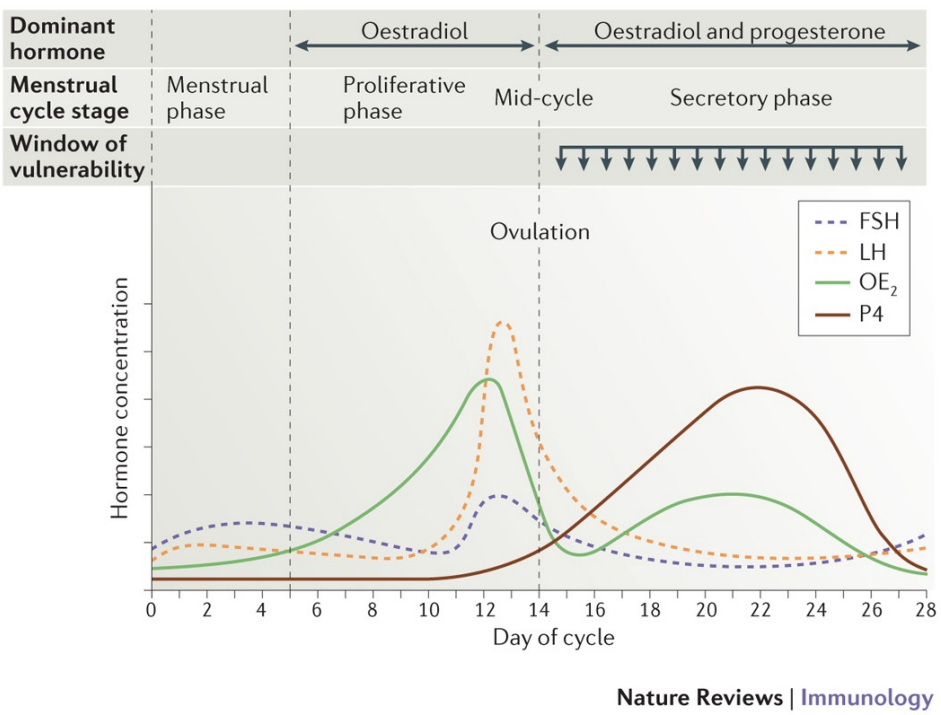

Figure 6.: Hormonal fluctuations during the menstrual cycle. The window of vulnerability indicates the period of the menstrual cycle when the woman is most vulnerable to infectious diseases. FSH = Follicle-stimulating hormone, LH = Luteinizing hormone, OE2 = Estradiol, P4 =Progesterone. Published with permission from Nature Reviews Immunology, Wira et al. [82]*, Copyright 2015, Springer Nature.*

8.5.3.2 *Pregnancy and infectious disease susceptibility*

The immune response towards pathogens in a pregnant woman is the result of combined signals and responses not only from the maternal immune system but also from the fetus and placenta as suggested by Mor & Cardenas (2010) [88]. The exact immunological alterations and physiological changes in the pregnant woman that make them more vulnerable to infections is subject to debate. However, both increased susceptibility and severity of infectious diseases have been noted for several agents (Table 2) [89]. Therefore, a clinical awareness and strategy for prophylaxis or vaccination of pregnant women is important.

Besides immunological modulation, pregnant women undergo several cardiopulmonary physiological changes (see section 8.2.3.1.1), which could make them more vulnerable to certain diseases, notably influenza and possibly COVID-19 [25,35].

Table 2. Infections associated with increased maternal susceptibility or severity during pregnancy, or severe adverse fetal outcomes (adapted from Abu Raya et al.*) [89].*

Increased maternal susceptibility	**Severe adverse fetal outcomes**
Listeriosis	Toxoplasmosis
Tuberculosis	Influenza
Malaria	Primary VZV infection
Increased maternal severity	Malaria
Influenza	Rubella
VZV Infection	Parvovirus B19
Hepatitis E Virus infection	Listeriosis
Malaria	Tuberculosis
Invasive *Haemophilus influenza* infection	Zika virus
Invasive pneumococcal disease	Measles
Invasive group A streptococcal disease	Mumps
Dengue Fever	Cytomegalovirus
Lassa Fever	
Ebola virus	
Primary Herpes Simplex Virus infection	
Coccidioidomycosis	
Measles	

8.5.3.3 *Aging and the immune response*

As a result of aging physiological processes in the body are undergoing a functional decline. Immunosenescence in both men and women manifests as a decreased ability to respond to pathogens, and the female advantage in mortality from infectious diseases is declining [1]. A drastic breakpoint in the epigenetic (including *inter alia* DNA methylation and acetylation) landscape in immune cells occur in both sexes, but at different ages in men and women. In men, an abrupt change occurs at age 62 to 64 years and in women 5-6 years later [26,90]. PBMC in older women have higher genomic activity for adaptive cells, while in older men, genomic activity is higher for monocytes and inflammation [90]. As Takahashi & Iwasaki (2021) [26] argue, a decline in activity for adaptive cells and increased activity in innate "blunt" proinflammatory gene expression, could make males more vulnerable to hyperinflammation and deficient adaptive immune responses.

8.5.4 ***Genetic (X linked) differences***

A difference in infectious disease incidence between pre-pubertal boys and girls, as well as between post-MP women and elderly men, indicates that not only sex hormones influence the immune response. Several reports indicate that sex chromosomes also affect sex bias in immune response [4,62,91]. The X chromosome contain about 900 genes compared to the smaller Y chromosome with its around 55 genes [92]. Several X-linked genes are involved in both the innate and adaptive immune system including the genes for TLRs and NFκB essential modulator (NEMO). TLRs are important pattern recognition receptors (PRRs) which sends early warning signals if pathogen-derived structures are encountered (section 8.3.1). NEMO modulates expression of NFκB (section 8.3.1.1), a transcription factor involved in multiple immune pathways. Additionally, about 10% of the total miRNA are also found on the X chromosome. miRNAs are involved in the translation and degradation of mRNA and therefore an important regulator of gene products [92]. The influence of sex hormones and genes on the X chromosome are considered to be independent of each other.

In females, one of the X chromosomes is randomly silenced early in embryonic development in a process called X chromosome inactivation (XCI), to allow dosage compensation of gene expression of XX females compared to XY males. This creates a mosaicism in the tissues where either the paternal (X_p) or maternal (X_m) X chromosomes is silenced and densely packed into Barr bodies. The remaining X chromosome will be available for transcription, and potentially half of the active X chromosome will be of X_p origin and half of X_m origin. This means that females have more variation in the transcribed X-linked genes as both gene products from X_p and X_m will be globally available. Having two copies of X

chromosomes, females are therefore less susceptible to X-linked mutations that result in disease in males.

Up to 15% of X-linked genes may escape inactivation to a variable degree depending on their location on the chromosome [92,93]. X-linked genes are very dosage sensitive and a disruption of the XCI mechanism in females will be detrimental and lead to tissue instability, tumorigenesis and decreased disease defense. Recently, autoimmune disorders (more often found in females) have been linked to perturbations of XCI [91,92].

TLR7 is located in a region on the X-chromosome which have a high chance of escaping inactivation leading to higher expression levels [92]. TLR7 is an important pattern recognition receptor (PRR) of the innate immune system. Exposure of PBMC to TLR7 ligands will cause a higher production of type I IFN (IFN-α) in female cells than male cells [3].

Patients with Klinefelter syndrome (KS) have an extra X chromones (47 XXY), resulting in low testosterone levels, high gonadotropins and estrogens. Their immune profiles have a more female phenotype (e.g. high $CD4^+$ count, high $CD4^+/CD8^+$ ratio, low $CD8^+$ count) and androgen replacement therapy may reverse some of these effects with decreased $CD4^+$ count, as well as lowered IL-2 and IL-4 levels [94]. Additionally, patients with KS have an increased risk of developing female predominant autoimmune diseases like Addison's disease, diabetes mellitus type 1, multiple sclerosis, acquired hypothyroidism, RA, Sjögren's syndrome and SLE [95].

8.5.5 *Behavioral and sociocultural aspects*

In many cultures, men are given better care within the family as well as outside [96]. Women's lower status also affect their ability to seek health care, their education level, and therefore their access to information on health and preventive measures. Being underinformed also affect the health of their children [96]. Cultural expectations on women also affect their health. For instance, women in Colombia waited longer for seeking health care than men when they contracted malaria, due to their responsibilities in the house hold [96].

Even in developed countries, women may receive substandard health care compared to men, due to unconscious bias [97]. Older women may be discharged earlier from hospital than older men who are not expected to be able to manage in a convalescent state in their home (personal observation). A Canadian study demonstrated, however, that men are less likely to seek their family physician for both physical and mental health concerns than women [41].

Particularly in low-income countries, socioeconomic and cultural factors may restrict women's access to health care and therefore cause undernotification of infectious diseases. Large systematic prevalence surveys are one way of avoiding undernotification of infectious diseases. One example of this is from Bangladesh where active TB-cases were investigated in a house-to-house survey. 51% of the 260'000 individuals included were men, however, the number of confirmed active TB cases had a male/female ratio of 3:1 [98].

8.6 Evolutionary drive for sex difference in immunity

The paramount goal, from an evolutionary perspective, of all species is to survive, reproduce and propagate offspring. In humans and most vertebrates, the mother is responsible for bearing the child and thus has the greatest responsibility [58]. A stronger immune response could thus be a way for the mother to brace herself for this task.

Resources (or energy) are finite and have to be allocated in a balanced manner to increase fecundity (mating success). The finite resources and their allocation will benefit males and females differently. Spending all resources on the immune response would not benefit propagation of genes for both sexes. To allow propagation of their genes, females, being restricted in number of offspring, would want to protect the few offspring produced and therefore allocate their resources to their immune response. Males, on the other hand, would want to mate as many females as possible (to allow higher propagation of genes) and therefore allocate resources to secondary sex characteristics. This would increase the chance of being selected by a female and also meet the competition from other males. For males this would mean less resources spent on the immune response. This would apply for a species where the sexual selection is high, i.e., where the males have to compete for being selected. This was first explained by Charles Darwin in his theory of sexual selection, which postulates that sex-specific phenotypes may spread throughout a species even if they are detrimental to survival, if they increase reproductive success [99].

However, being infested with a parasite could be detrimental for the attractive display (including, e.g. ornate tail feathers, impressive antlers and advanced courtship) and could leave the male with zero success in mating. The male would therefore need to balance his finite resources on both male traits and immune response (Fig. 7).

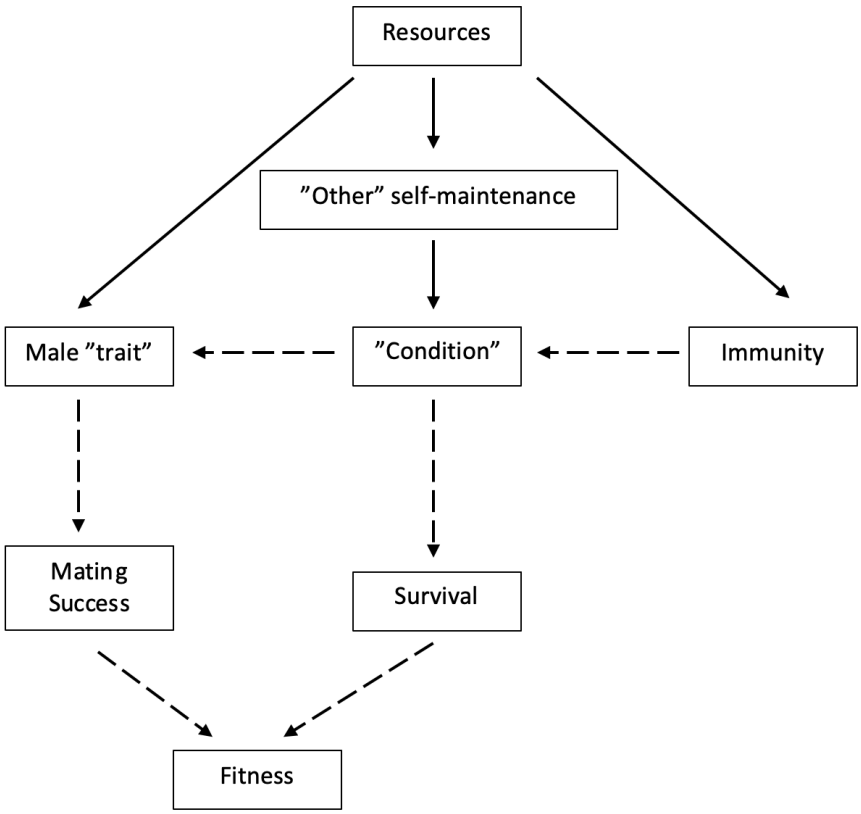

Figure 7. (Adapted from Zuk&Stoehr[100]). Allocation of finite resources according to a simple model. Resources may be allocated to promote immunity to the benefit of male "traits", i.e. traits that will increase their mating success. Some of the resources do also need to be spent on maintaining a physical "condition". This "condition" will determine both the probability of survival and be a necessary requirement for male "traits". Shown here is the male case. In females the reproductive effort is simply fecundity.

9 Aims of the thesis

The aim of this thesis was to explore sex differences in the immune response to infectious diseases, and more specifically to:

1. Gain further insights in how sex hormones influence the immune system in healthy individuals.
2. Evaluate the expression of SHR in immune cells during the menstrual cycle in healthy pre-MP women.
3. Add more information to the expression of SHR in specific immune cells separated by FACS.
4. Investigate expression of immune markers in immune cells during the menstrual cycle (in healthy women) and if these markers can be linked to the levels of hormones and/or SHRs.
5. Investigate the expression of SHRs in PBMC from patients with HFRS during the acute and convalescent phase of the disease.
6. Investigate the presence of TTV in PBMC from healthy individuals, and whether the presence and levels of TTV is linked to hormones or hormonal aberration.

10 Methodological considerations

Scientific hypotheses may be tested on a macroscopic level focusing on e.g., public health, or on a (sub-)microscopic level in molecular studies. The methods used depend on the type of data generated (e.g., epidemiological data, interviews, clinically controlled trials, etc.) This thesis is based on results from experimental studies on nucleic acids on a small number of subjects. Epidemiological studies including thousands of subjects will naturally have more statistical power and are more reliable in terms of statistical evidence. Experimental studies will inevitably have to performed initially in a small scale to explore novel ideas and hypotheses. However, there is a risk of misinterpreting, overestimating or missing out on interesting findings when a small number of subjects are included.

In this section I will describe the use of the respective methods and why it was chosen in this project.

10.1 Methodological considerations when studying sex differences

Historically women have been underrepresented in medical research, which previously solely was based on (young) male participants. Unfortunately, this has led to erroneous conclusions as results from these studies regarding pathogenesis and treatment strategies have been applied to include the whole population. The Swedish Research Council, one of the main funding sources of research in Sweden, now requires a sex and/or gender perspective in their applications and define the two terms accordingly: "*Sex describes the division into categories based on biological characteristics, usually women and men, girls and boys, male and female animals. Gender describes, in simple terms, the social and cultural processes that construct perceptions of sex and has implications for both structures of society and the gender identity of individuals*." In this thesis, sex is used in its biological sense and sex differences are applied to physiological differences, including genetic differences. Applying these terms strict and not confusing the use of gender/sex is advised for consistency in the literature.

Studying sex differences and discerning their mechanisms in biological systems is complex and requires thought on the methodological setup. In laboratory animals, knock-out models have been created with deletion of *ESR1* generating ERα knock-out (ERKO) or *ESR2* (ERβ knockout, BERKO). ERKO mice are not fertile and the global *ESR1* deletion will also result in high testosterone levels (which in turn give rise to other effects downstream of AR signaling) [101]. BERKO

mice will have reduced fertility and do not show the same phenotypically grave deficits as ERKO mice. The deletion of a complete receptor gene will also have effect on non-immune cells in ways that may impact the immune response [101]. Gonadectomy of young mice and replacing estrogens and androgens with slow releasing hormonal pellets is another approach. However, the absence of sex hormones may alter the development and differentiation of immune cells before pellet implantation [101]. Further, the contribution of cyclic variation of hormones in female mice may not be replaced by a constant release from hormone pellets [101]. While studying cell cultures alone may allow full control of the impact of hormones on the respective cells, the results from these types of studies do not provide complete answers to the regulation *in vivo* [101].

One challenge faced when studying physiological sex differences is to elucidate whether the difference is dependent on genetic mechanisms (sex chromosome complement) or related to sex hormones. One clever model to circumvent this problem is the "four core genotypes", FCG. In this model, XX and XY males, as well as XX and XY females are produced. In these mice, the testis-determining gene *Sry* is deleted from the Y chromosome (XY$^-$), which rendered the XY$^-$ mice as gonadal females. Sry inserted onto an autosome produced XX*Sry* males. This allows comparison of XX females and XY- females with XY males and XX*Sry* males [102].

10.2 Considerations on study design

As a medical doctor, I have an interest in the clinical picture and evaluation of patients. This is the main reason that I chose to work with human material. For me, the interesting part is when results from research may be applied in a clinical context.

Working with human samples is demanding and requires: (1) Accessing the human samples, (2) careful ethical considerations, (3) sufficient power in the data analysis, which may be challenging considering the numbers of patients needed to recruit, (4) confounding factors including environmental factors (e.g., diet, smoking, occupation and social, cultural, gender and geographical factors), and physiological factors (genetic diversity, differences in BMI, age, sex and comorbidities) and (5) differences in timepoint of patient inclusion (which day of onset of illness). The obvious strengths working with human material are that the findings may be directly applicable. Even though man and mice share 98% of the genome, previous findings in research animals have failed to be applicable to humans. For instance, corticosteroids are teratogenic for animals but not in humans, while thalidomide is teratogenic in humans but not in mice [103].

Studying sex differences, multiple interesting findings have been reported from experimental studies. Especially in research regarding sepsis and sex differences, interesting findings in rodents have been reported. However, many of these findings have failed to be extrapolatable to humans [39,103]. This is another reason to why studies on human samples should be performed.

10.2.1 *Detection of ER in samples*

Working with mRNA, one should consider that not all mRNA transcribed are further translated into peptides. So, mRNA-levels do not necessarily reflect the amount of protein in the end. From this point-of-view, detecting the protein itself would be preferrable to eliminate the influence of further regulatory steps on an mRNA-level. However, investigating proteins is trickier than handling nucleic acids, as proteins are less stable and harder to detect, while mRNA can be reverse-transcribed to cDNA, which in turn can be amplified in the qPCR reaction. Methods for specific protein detection are also dependent on specific antibodies, which in the case of ERβ is debated [104]. As with every test procedure, there is a subtle balance between specificity and sensitivity. Ideally the procedure should detect *all* protein of interest (100% sensitivity) and *only* the protein of interest (100% specificity). Antibodies for ERα are recognized as specific, and multiple variants exist from several producers. For ERβ1, although several variants are produced, their specificity have been questioned [104].

Furthermore, there are no antibodies commercially available selective for ERβ2 (that differ to ERβ1 with 36 amino acids), and the ones used experimentally are made in-house at specific laboratories. Due to the limitations in ERβ1 and ERβ2 specific antibodies, I chose to use the more reliable and easily quantifiable detection of ER mRNA by qPCR.

10.3 Included methods

10.3.1 *Manual qPCR*

This method was used in paper III and part of paper I. In paper I, cells separated using FACS (see separate section below) were prepared for quantitative PCR (qPCR). Primer pairs for ERα, ERβ1, ERβ2, AR and PR were applied. For every qPCR, a reporter gene must be amplified to use as a reference to the gene of interest (GOI). The reporter gene should be constitutively expressed to avoid misinterpretation and therefore chosen with care. A suitable reporter gene in one

tissue could show fluctuating activity in another. In our study on PBMC, using manual qPCR, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was chosen as a reporter gene and samples were run in duplicates to minimize technical variation.

The advantages of using manual qPCR are the flexibility and the possibility to switch to other reagents and primers depending on yield and success-rate. qPCR as a method is quite unproblematic as improved reagents and more advanced master-mixes have developed - a step forward from the previous handling of multiple reagents which may increase the risk of technical variation. The MIQE guidelines first published in 2009, are striving to support consistency and quality in the publishing of experimental results from qPCR, and should be followed in planning, performing and publishing results from qPCR experiments [105].

Disadvantages using manual qPCR (as compared to array-based methods) are that the evident risk for technical variation due to handling small volumes and multiple reagents.

10.3.2 *qPCR Array, TaqMan low-density array (TLDA)*

In paper I, a TaqMan low-density Array (TLDA, ABI Foster City, CA) was used. The array cards were designed in Format 32 (31 genes+ control) and preloaded with primers for the respective genes. In the array, samples were run in triplicates, and GAPDH was used as a reference housekeeping gene.

Strengths using TLDA is includes minimizing technical variation in the samples as the primers are preloaded. The template is applied in one of the 8 sample loading-ports on one side of the array card and distributed to the 48 connected wells through centrifugation. The centrifugation process assures the distribution of a similar amount of template in each well.

This also means that less template is needed to perform the analysis. Drawbacks with this method are the higher costs and a fixed gene set-up which makes follow-up analyses harder to perform.

10.3.3 *Fluorescence-activated cell sorting (FACS)*

FACS is a technique that allow sorting of cells very specifically using laser beams that sort cells bound with specific fluorescent antibodies. It allows analysis of specific cell types but requires living cells and is therefore sensitive to previous handling of the material. Even though correctly handled, cells are sensitive to storage time in the freezer. FACS is also an expensive and highly time-consuming analysis. The costs of reagents and running cost for the cell-sorting machine naturally causes restraints to the possibilities this technique offers. This

technique was applied in paper I to investigate the presence of SHR mRNA in specific PBMC cell subsets.

10.3.4 *R-Gene qPCR viral quantification*

In Paper III, a TTV amplification Argene TTV R-gene® (bioMérieux S.A., Marcy l'Etoile, France) kit was used on a real-time qPCR system (ABI). The kit includes TTV DNA plasmids corresponding to 5, 50, 500 and 5000 virus particles per µL, as well as a sensitivity control containing 1 viral DNA/µL. This allows a setup of an internal quantification standard which simplifies quantification. The sample wells were run in triplicates using 10 µL of enriched DNA solution. The detection limit was set to >1 viral particle in the sample reagent (10µL). A sample was considered positive if 2 of 3 triplicate samples were above the detection limit.

This specific kit is CE-marked [106], and has successfully been used in previous studies on transplant patients to evaluate their risk of developing iRAE [107]. Using a kit with verified performance stability is essential for a reliable comparison of research results, and previous disparate analyzing methods hampered the development in this field [22]. Historically, investigating the presence and levels of TTVs in the blood have been demanding due to the high genomic variability of TTVs, with 29 described species. Of these 29, 12 are species considered more abundant [108]. The kit used in our experiments covers all of these species [106].

The benefit of this kit is the simplicity of use and the construct of universal primers which includes detection of multiple TTV species. The obvious drawback is the high cost per kit. However, the cost for equipment which simplifies laboratory work should be seen in relation to increasing number of man-hours in the lab, and the risk of less reliable data sets based on previous methods.

10.4 Statistical analyses

Classical statistical tools were used to estimate the variation between two groups (Welch's t-test) and paired t-test (if the groups were paired). Repeated measures correlation was used to calculate the correlation coefficient for paired samples, and t-test to test the significance of the correlation coefficient. A Bonferroni adjustment was used to avoid inflation of Type I error.

Analyzing several parameters at the same time requires multivariate statistics. A binominal regression analysis was used in paper II. The regression model was analysed using software R 3.6.0 and RStudio 1.2.1335. dplyr 1.0.2 was used for data processing. In paper III, the multiple variables from the blood chemistry,

relative expression levels of ERs mRNA, and day of onset of the disease were related to each other in a principal component analysis (PCA) (SIMCA-P, v 12.0.1).

11 Results

Here follows a summary of the results from the three papers included in the thesis. For details, please refer to the original paper included below.

11.1 Paper I (Expression of sex hormone receptor and immune response genes in peripheral blood mononuclear cells during the menstrual cycle)

In this paper we explored the presence of SHR in PBMC from healthy individuals using manual qPCR and TaqMan low-density array (TLDA). From PBMC, cells were sorted into T-cells (CD4+ and CD8+), NK cells (CD56+), monocytes (CD14+) and B-cells (CD19+). In the TLDA analysis, we included SHR and several immune markers (see appendix) on 10 pre-MP women longitudinally sampled (4 times each) through the menstrual cycle.

11.1.1 *Manual qPCR and ER mRNA expression*

ER mRNA was present in all investigated cell types; ERα was most abundant, but the truncated isoform ERβ2 was found in higher concentrations than (wild type) ERβ1. In most cases ERα was most abundant followed by ERβ2 and ERβ1 in CD4+ and CD8+ T-cells and NK-cells. B-cells contained similar amounts of ERα, ERβ1 and ERβ2. Of all investigated ERs, ERβ1 was found in lowest amounts and it was concentrated to $CD19^+$ B-cells. Other cell types had no or very small amounts of ERβ1 expression. ERβ2 was found to be the dominating ERβ isoform. ERβ2 was mainly found in B-cells but also in higher amounts than ERβ1 in T-cells and NK-cells.

11.1.2 *TLDA on pre-MP women through the menstrual cycle*

The results from this analysis showed no difference in SHR expression comparing the different phases of the menstrual cycle. However, several immune related genes differed significantly comparing mid follicular with ovulatory or mid luteal phases and in the case of *IFNG* both during early follicular and mid luteal compared with the ovulatory phase.

As expected, several immune related genes were correlated in their expression. This is probably related to coregulation of genes, as genes are seldom solely activated, rather a group of genes are activated together. Further, correlation of

gene expression of immune related genes with hormone levels was analyzed, including E_2, FSH, LH, P_4, and T as well as SHBG. Notwithstanding, no significant correlation (α = 0.00046) could be noted for any of the included hormones, P_4 stood out with high r-numbers and/or low *p*-values for several genes (*STAT5A, TGFB1, STAT3, GATA3, IL1B, TNFA, TBX21* and *IFNG*). The close to significant correlation to P_4 is interesting as we could not detect expression of the progesterone receptor (PGR) in our material, a finding also noted by others [109]. This suggests an alternative pathway of P_4 regulation, possibly through the GR [109].

Interestingly, *ESR_ERB1* (ERβ1) was significantly positively correlated to sex hormone binding globulin (SHBG). Previous studies have shown opposite correlation of SHBG and E_2. We speculate that ERβ1 could oppose the action of ERα signaling elicited by E_2.

1.6.1.1. Gene expression during the menstrual cycle

We could not detect any differences in SHR (AR, PGR, ESR1, ESR2-ERβ1 or ERS2-ERβ2) expression. However, the genes *GATA3, IFNG, IL1B, LTA, NFKB1, PDCD1, STAT3, STAT5A, TBX21, TGFB1, TNFA* showed all significantly different expression during the menstrual cycle.

IFNG, TNFA and *IL1B* are all genes that encode proinflammatory responses. NFKB1 encoding the transcription factor NFκB (see section 3.4.1.6) is a transcription factor that may be induced by steroid signaling. A fluctuation in expression related to the menstrual cycle is therefore not unexpected, although causality is not proved in this paper.

GATA is a family of transcription factors that bind to a DNA-sequence containing the nucleotides "GATA". GATA-binding protein 3 (*GATA3*) and T-box transcription factor 21 (*TBX21*), encoding for T-bet, are important factors in the differentiation of T_H1 and T_H2-cells respectively. Previous studies have reported a shift in T_H1-T_H2 response during the menstrual cycle [62]. We cannot repeat this finding as both *GATA3* and *TBX21* are highly expressed during the same phases (ovulatory and mid luteal).

The same expression pattern is seen for *PDCD1*, *TGFB1*, *STAT3* and *STAT5A*. *PDCD1* and *TGFB1* are both involved in immune tolerance. TGF-β (endcoded by *TGFB1*) stimulates differentiation of $CD4^+$ T-cells to T-reg cells, and *PDCD1* encodes for PD-1, an immune checkpoint that may induce or repress apoptosis in immune cells.

Signal transducer and activator of transcription 3 (STAT3) and STAT5A were both highly expressed during the latter part of the menstrual cycle. STAT3 has been associated with bacterial infections and viral infections with hepatitis B (HBV), HCV and human papilloma virus (HPV). These viruses are all known to promote the development of cancer. HBV and HCV may induce hepatocellular carcinoma (HCC), and HPV cervical and rectal cancer [110]. In previous reports, STAT5 have been associated to sex bias noticed in liver metabolism [111] and pulmonary hypertension [112].

11.2 Paper II (Blood hormones and torque teno virus in PBMCs)

In this study we investigated the presence of the commensal TTV in PBMC from healthy individuals. Overall, the prevalence of TTV was higher in men (50%) and post-MP women (25%) compared to pre-MP women (17.6%). In pre-MP women we noted that all positive individuals were either anovulatory or hypothyroid. No men or post-MP women were hypothyroid.

Of pre-MP women, TTV^+ individuals also had significantly lower progesterone (P_4) levels than TTV^-. Generally, P_4 has an immunosuppressive effect, and the *increased* levels of P_4 together with *lower* levels of immunostimulatory E_2 during the luteal phase characterizes the "window of vulnerability" (see section 3.6.4.1 and fig "hormonal fluctuations during the menstrual cycle"). Increased P_4-levels are therefore expected to be associated with vulnerability to infections. In the literature, studies point in both directions, with increased risk at *high* P_4 levels for some agents (tuberculosis, HSV and HIV), and increased risk at *low* P_4-levels (influenza, *S. typhimurium* and *C. difficile*). As the regulation of the immune system *in vivo* is complex, possible reasons for this contrast may be: (1) Each infectious agent stimulates the immune response uniquely. P_4 could promote or demote this response depending on its components. (2) Other (unknown or not analyzed) factors could elicit a stronger signal that outplays the effect of P_4.

11.3 Paper III (Gene expression of ER in PBMC from patients with Puumala virus infection)

In this article we retrospectively investigated the expression of ERα, ERβ1 and ERβ2 genes in PBMC together with the blood chemistry (creatinine, CRP, TPK, urea, etc.) in patients with HFRS, twice during the acute stage of the disease and again in the convalescent phase. ERα and ERβ2 were both expressed in PBMC,

ERβ1 was found in little or no detectable amounts. Using a principal component analysis (PCA), we concluded that female and male participants had different a different profile of total lab results. In the PCA, female samples were more associated to an increase in ERα, and male samples to an increase in ERβ2.

Furthermore, in the gathered clinical data, we noticed that male patients needed stronger analgesics than female patients (unpublished data). This finding is interesting, but conclusions on whether this is the results of different immune response or behavioral factors (e.g., caretaking of male versus female patients) are hard to draw.

12 Discussion

In this thesis I describe the expression of SHRs, immune related genes and Torque teno virus in PBMC in three different papers. Expression in PBMC of SHR mRNA and detection of SHR proteins have previously been described in other small studies [49,50]. However, paper I is the first study to (1) estimate the expression of mRNA of ERβ2 in separated immune cells and (2) to describe the expression of SHR through the menstrual cycle.

In paper I, ER expression is differentiated into ERα, ERβ1 and ERβ2. The results therein indicate that ERα is most abundant in all investigated cell types. In $CD4^+$ T-cells, $CD8^+$ T-cells and $CD56^+$ NK-cells, higher expression of ERβ2 is noted compared to ERβ1. Monocytes (CD14+) have no or very low expression levels of both ERβ1 and ERβ2, while CD19+ B-cells have similar levels of both ERα, ERβ1 and ERβ2. In a previous similar, well-cited study, also performed on separated cells from PBMC [49], qPCR-primer pairs that covered *total* ERβ was used, which does not distinguish between isoforms. It is therefore possible that, wildtype ERβ1 was modestly expressed even in this material compared to other isoforms, including ERβ2.

As described above in the background section, ERs hybridize to dimers in order to reach their active state. Interestingly, ERβ2 is believed to function as an antagonist of ERα-signaling by having a dominant negative effect on ERα when dimerizing [113]. ERβ2 has lost its ligand-binding capacity, so its main function is probably to interact with other ERs as one part of the dimer pair. The propensity to dimerize is αβ2>β1β2 [113] and also β1β1>β1β2 [77]. ERβ2 homodimers are not formed [77]. This implies that the available ERβ2 would preferably bind to ERα (rather than ERβ1) and activation of E_2-ERα mediated gene transcription will be hampered. Instead, this would leave an opening for ERβ1-regulated signaling.

As ERα and ERβ have similar affinities for E_2, and the general opinion is that the ratio ERα/ERβ in the cell will decide the downstream signal and eventual gene transcription. However, as described above, the amount of ERβ1 and ERβ2 will also affect activation of ERα or ERβ-regulated genes.

Accordingly, knowledge of ER isoform expression is important for understanding intracellular gene expression. A possible function of ERβ2 in healthy pre-MP women could be to act as a buffer to dampen the E_2-ERα signal as E_2-levels varies during the menstrual cycle.

Further, we report in paper I that ERβ1 is predominantly expressed in CD19+ B-cells, and the amount of ERα, ERβ1 and ERβ2 in these cells is similar. The effect of ER-ERβ1 signaling should therefore be more prominent in B-cells compared to other cell types investigated in this study were the amount of ERβ1 was low. As E_2 is higher in pre-MP women, sex-specific effects in B-cells could therefore be related to ERβ1. Indeed, E_2 have been correlated to several effects in B-cell maturation and selection, as decreased lymphopoiesis in the bone marrow, altered B-cell subsets in the spleen and decreased b-cell receptor signaling [114].

B-cells are the source of antibodies (immunoglobulins), and women are known to produce more antibodies than men, considering both basal levels [115] and as a response to vaccination [3]. Production of multiple autoantibodies is a hallmark of disease development of SLE. Interestingly, disruption of ERα attenuates progression of SLE in a murine SLE model [116].

In ER expression analyses based on patients with NE (Paper III), again ERβ1[†] was found in very small amounts. ERβ2 was, also in this material the dominant form of ERβ. Further, in paper III we note, that samples from women and men are differently grouped according to laboratory profile. Male samples are according to multivariate analysis (PCA) more correlated to ERβ2 and female samples are more correlated to ERα.

Several articles have investigated the role of ERβ isoforms in cancer and found that ERβ2 and ERβ5 have protective properties, whereas ERβ1 promote tumorigenesis and progression of cancer [117,118]. A similar opposing pattern could be relevant in infectious disease pathogenesis.

In the results from the TLDA-array in paper I, significant differences in expression were noted for several immune related genes depending on the phase of the menstrual cycle. One of these is *PDCD1*, encoding for the protein PD-1. *PDCD1* expression was found to be significantly more expressed during the ovulatory and mid-luteal phase compared to the mid follicular. PD-1 is an immune checkpoint protein that may induce or repress apoptosis in cells, and PD-1 inhibitors (anti-PD-1) have recently been identified as targets for several types of cancer, e.g. melanoma. According to a meta-analysis of the effect of checkpoint inhibitors, the effect of anti-PD-1 seems to be more efficient in men [119]. As shown in our paper I, expression of *PDCD1* is related to the phases of the menstrual cycle, making it likely to believe that it is regulated by sex hormone

[†] In paper II, ERβ1, is referred to as ERβ. To avoid confusion, the term ERβ should preferably be used when referring to total ERβ, and the isoforms should be referred to using their specific designation (ERβ1, ERβ2, etc.).

levels. The lowest expression levels of PDCD1 were noted in the mid-follicular phase (when E2-levels peaks), while the highest expression levels were noted in the ovulatory and mid-luteal phases (when E2-levels are low).

Several studies have addressed treatment of COVID-19 and focused on 1) viral suppression in the early stage of disease and 2) immunosuppression in the later stage of disease, resulting in treatment regimens including remdesivir and cortisone, respectively. Recently an interesting randomized clinical on the immunosuppressive IL-6R antagonist tocilizumab was released as a pre-print [120]. In the results from this paper, an interesting finding was noted. There was a statistically significant survival benefit for individuals that received cortisone + tocilizumab compared to the group that received cortisone only. However, the positive effect of adding tocilizumab was only valid for males, not for females [120]. This result is not surprising in the light of previous studies on immunotherapies. Generally, immunotherapies that promote immunity are more effective in females and immunotherapies that dampen immunity are more effective in males [121].

The different sex hormonal profile between men and women could be the reason for the high degree of asymptomatic women with NE. Before puberty, NE cases are scarce [10]. In a nationwide survey of all reported pediatric cases of NE in Sweden 1984-1992, merely 32 patients < 15 y.o. were identified [122]. This implies that either children are less exposed to PUUV, or that their immune response differs from the response in adults. As the morbidity and mortality from multiple ID is increasing after puberty, interactions of sex hormones with the immune response to hantavirus is plausible [1,123]. In the survey by Ahlm *et al.* (1994) [122], they report a sex difference among their pediatric patients by boys/girls 1.7/1. The numbers of cases (n=32) are limited, but a pediatric sex difference could be explained by genetic factors, and a higher incidence among adults by sex hormones. If sex hormones are contributing to in the sex bias, one could hypothesize that ERβ isoforms are be involved in determining the degree of symptoms between men and women. The tendency in our analysis of female samples being more correlated to an increase in ERα and male samples to ERβ2 could explain the sex differences in immune response noted by others [11], and therefore and the symptomatic picture – in the bottom line the result of your immune response.

Detection of Torque teno viral DNA in PBMC is described in this thesis. In paper II, a higher prevalence of TTV in PBMC was noted in men and post-MP women compared to pre-MP women. Interestingly, all the TTV^+ pre-MP women were

hormonally aberrant, i.e. anovulatory and/or hypothyroid. Hypothyroidism is generally not regarded as immunosuppressive but deviating sex hormones (resulting in anovulation) could certainly affect the immune response. Lower female sex hormones will lead to a more male immunophenotype in pre-MP women, with increased risk for infectious diseases. Post-MP women, with naturally lower sex hormones, did also have a higher prevalence of TTV. Although most likely, both low female sex hormones and age-related functional decline of the immune system (i.e. immunosenescence, see background section), contribute to this effect.

The connection of TTV to hormonal status contributes to the notion that hormones interact with the immune response. Traditionally, we have been categorizing pathogen response into immunology and infectious diseases, and hormonal influence into endocrinology. The reality is, not surprisingly, far more complex.

13 Concluding remarks and future perspectives

In conclusion, the results in this thesis reveal distinct patterns of immune response related to sex hormone levels, SHR expression and the phases of the menstrual cycle, supporting that there a link between sex hormone levels and immune responses. Moreover, we show that the ER isoform ERβ2 is more abundant in PBMCs than what was previously described. The results in this thesis lends further credence to sex hormones being contributory factors to sex bias in immune response and exemplifies the importance of taking these differences into account in a clinical setting.

Studying sex differences will save lives and money. Women have more frequent side-effects following drug and vaccine administration as historically, dosage have been evaluated in men only. Tailoring the treatment is particularly important for long-term and chronic treatment as in life-long medication of HIV, where women more frequently report more side-effects and sex differences have been noted for several types of drugs (eg, NRTIs, NNRTIS and PIs). The discrepancy in outcome between men and women in immunomodulatory treatment of both cancer, autoimmunity disorders and infections [120,121], underlines the importance of taking sex and hormonal status of the patient into consideration when designing research studies and in clinical practice.

By frequently reporting data from both men and women in scientific reports the data on sex differences will increase the current knowledge and possibly lead to a more personalized medicine – to the benefit of both men and women.

13.1 ER and immune marker expression

A potential continuation on this project would be to include pre-MP women and sample $CD19^+$ B-cells specifically, to estimate ERβ1 and ERβ2 mRNA expression levels and investigate whether these isoforms fluctuate in relation to the menstrual cycle. Unfortunately, this evaluation was not possible in our collected material due to limited number of participants. Such analysis could be performed using magnetic separation of cells (e.g. Dynabeads®) rather than by using FACS. Using magnetic beads to separate a specific cell type is a less laborious and faster method than FACS once you have decided which cells to analyze. The selection

may be either positive (selecting for the cells of interest) or negative (removing unwanted cells, leaving cells of interest untouched) and includes biotinylated antibodies which reacts with streptavidin-coated magnetic beads. Similarly, it would be interesting to study the effect of ERβ1 and ERβ2 expression on B-cells activated by an antigen with particular sex bias (e.g. Hantavirus and SARS-CoV2).

As mentioned above, STAT5 and STAT3 have previously been linked to sex differences in the pathophysiology of pulmonary hypertension and liver metabolism. Additional studies including samples from infectious disease patients or antigen-activated cell cultures are warranted to elucidate the possible role of STATs in the immune response towards pathogens.

It would be interesting to study the expression of the glucocorticoid receptor (GR) in PBMC and the possible effect of P_4 on these cells. In my collaboratory group in Huddinge, we are planning to perform additional experiments on GR.

13.2 Torque teno virus

Further research on TTV has immense possibilities as of the now commercially available test. There are multiple conditions with immune suppression that could benefit from a better mapping regarding immune status. Results from studies on transplanted patients mentioned above demonstrate the practical use of TTV to evaluate immunosuppression in a clinical setting, which could be of use in predicting graft rejection and risk of opportunistic infections. The possible application of TTV should preferably be further evaluated together with currently available methods for estimating the immune response.

The high daily turn-over rate for TTV (see background in paper II) allows detection of rapid changes in the functional immune response based on TTV-DNA quantification. This should be performed on serum rather than PBMC, as the levels in serum appears to be higher, as we report in paper II. A profound analysis of TTV-DNA quantification during the menstrual cycle would be appealing to evaluate the immune status according to the hormonal milieu. Further, infectious diseases which are exaggerated by a decline in immune response (activation of LTBI, shingles, herpes, etc.) are other possible conditions where TTV-levels could prove to be a valuable tool in the clinical evaluation.

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