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Genital Chlamydia Infection is Influenced by the Female Sex Hormones Estrogen and Progesterone in Vivo

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ABSTRACT

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by

Amy Gravitte

Chlamydia is the most common bacterial sexually transmitted infection in the United States and worldwide. It often goes unnoticed due to lack of symptoms and left untreated it can ascend the female genital tract to cause sequelae like pelvic inflammatory disease and irreversible tubal infertility. In reproductive-aged women, female sex hormones estrogen (E2) and progesterone (P4) concentrations fluctuate during the menstrual cycle and are influenced by hormonal contraceptives and hormone replacement therapy. E2 and P4 influence genital *Chlamydia* infection in women and mice, but these multifactorial interactions are not entirely mapped out. The complex interplay of E2 and P4 with *Chlamydia* and the host response demand further study to determine the effect of hormonal environment and host susceptibility to *Chlamydia*.

E2 primarily signals through estrogen receptors (ER) ERα and ERβ. We used ERα or ERβ knockout (KO) mice to study the role of E2 and ERs in chlamydial progression and examined the host immune response at day 9 post-infection, when we expected the immune response to be the most robust. ERαKO, but not ERβKO mice had significant differences in the progression of *Chlamydia* and the host immune response. Future studies should test the immune response at additional timepoints, and a model should be utilized wherein ERα and ERβ are simultaneously silenced by chemical knockdown of ERβ in ERα knockout mice using ER agonist ICI 182, 680.

Mice are widely used in *Chlamydia* research, but due to its short estrus cycle, infection cannot be established naturally before infected cells are shed. To overcome this, mice are pretreated with depot medroxyprogesterone acetate (DMPA), an exogenous progesterone that halts the estrus cycle. However, a mouse model not reliant on DMPA pretreatment is needed because 1.) DMPA can affect the immune response and 2.) the hormonal environment in women is not static. Our model uses mice that are ovariectomized to stop the production of endogenous E2 and P4, then treated with physiologically relevant levels of E2 and P4 via implantation of a hormone-filled capsule. We observed that E2 protected mice from *Chlamydia,* making our model a good alternative for *in vivo Chlamydia* studies.

DEDICATION

This dissertation is dedicated to my husband, Matt, who has been there for my highest highs and lowest lows and loved me through them all. To my dad, who helped me with my homework from the first day of kindergarten and pushed me to be my best. When I'm not sure of myself, you always are. We did it! To my mom, who knows me to my core, knows when to give me a push and when to let me cry. Your talks and phone calls boost me more than you could imagine. To my friends, who help me let go, relax, and live a little (or a lot) and make me laugh when I need it the most. And to our Creator, who provided me with them all.

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CHAPTER 1. INTRODUCTION

Genital Chlamydia

Genital *Chlamydia* is a sexually transmitted infection (STI) caused in humans by *Chlamydia trachomatis*. It is the most common notifiable bacterial STI in the US, with nearly 2 million new cases reported in 2019 alone according to the Center for Disease Control's (CDC) Sexually Transmitted Disease Surveillance. *Chlamydia* also dominates as the most common bacterial STI worldwide (Rowley et al. 2019). Symptoms of genital *Chlamydia* may include odorless vaginal discharge, cervicitis, and dysuria in women, and urethritis/urethral discharge in men (Malhotra et al. 2013; Shetty et al. 2021). *Chlamydia* infection is treated using antibiotics, typically a regimen of azithromycin or doxycycline (recommended by the CDC). However, an estimated 77-94% of genital *Chlamydia* cases are asymptomatic (Farley et al. 2003; Korenromp et al. 2016), and therefore go unnoticed and untreated. Untreated genital *Chlamydia* infection can be detrimental to women's reproductive and sexual health. As *Chlamydia* reproduces, it ascends the female genital tract (Figure 1.1) and causes severe sequelae that can be irreversible, including hydrosalpinx, pelvic inflammatory disease, ectopic pregnancy, and tubal infertility (Malhotra et al. 2013; Price et al. 2013; Ljubin-Sternak and Meštrović 2014; Shetty et al. 2021).

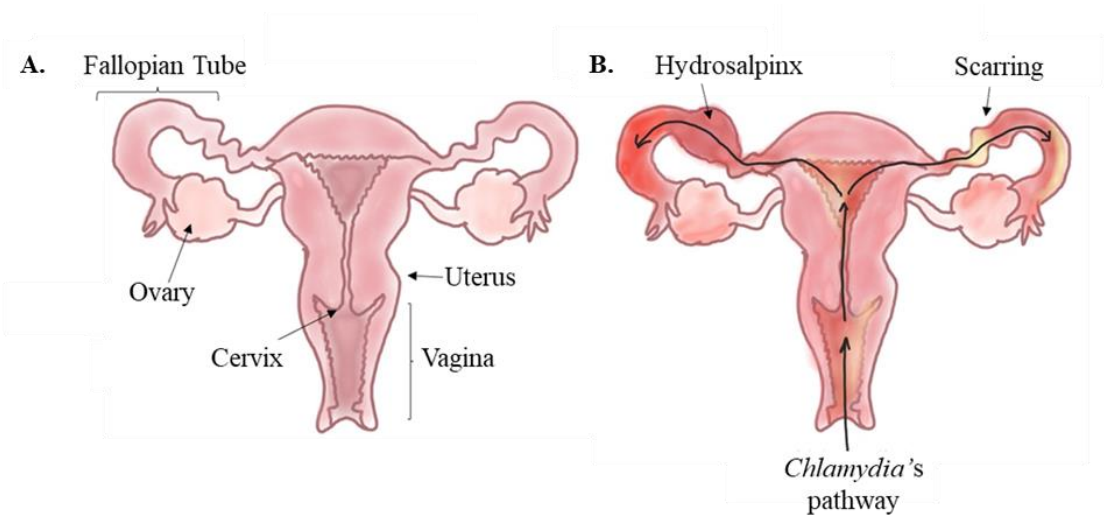

Figure 1.1: *Chlamydia* in the Female Genital Tract. A. A healthy female genital tract. B. An infected female genital tract depicting *Chlamyida's* pathway and pathologies including hydrosalpinx and scarring.

Chlamydial Development

Chlamydia trachomatis is one of 11 identified species in the *Chlamydia* genus (Bachmann et al. 2014). Members of this genus are Gram negative obligate intracellular pathogens that primarily infect mucosal epithelial cells and undergo a unique biphasic life cycle consisting of two morphologically distinct forms: the elementary body (EB) and the reticulate body (RB) (Wyrick 2000) (Figure 1.2). EB are the small (0.3μm), electron dense, metabolically inert, infectious form of *Chlamydia* that are bound by a rigid cell wall. RB are the larger (1.0 μm), granular, non-infectious form of *Chlamydia*. They are bound by an inner and outer membrane, and replicate via binary fission (Becker 1996; Abdelrahman and Belland 2005). The exact mechanism of chlamydial attachment and entry into host cells has not yet been defined, but several *Chlamydia* and host proteins have been implicated in the process. Known bacterial proteins involved in EB-host cell entry include type three secretion system (TIIISS) (Nans et al.

2014) and the chlamydial major outer membrane protein (MOMP) (Su et al. 1990). Known host proteins include human epidermal growth factor receptor (EGFR) (Mölleken et al. 2013), ephrinA2 receptor (EphA2) (Subbarayal et al. 2015), and protein disulfide isomerase (PID) (Natalia V Guseva et al. 2003; Conant and Stephens 2007).

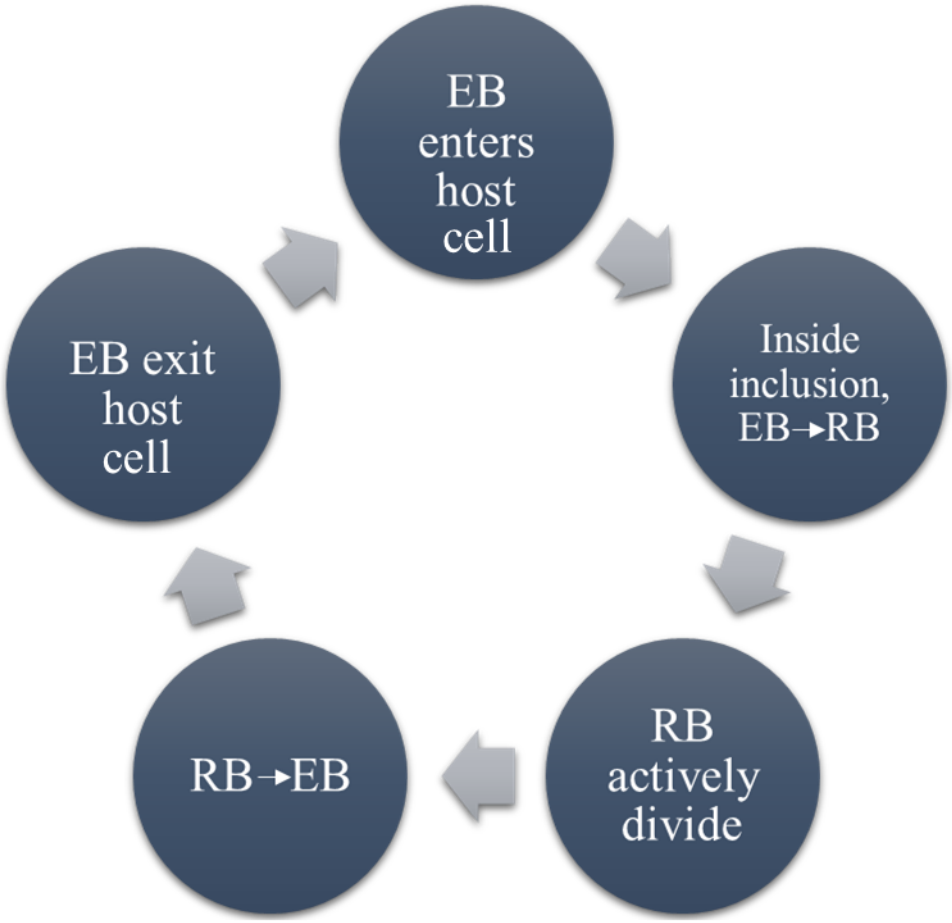

Figure 1.2: *Chlamydia* reproductive cycle

Once inside a host cell, EB reside inside a vacuole-like space called an inclusion, where they undergo primary differentiation into RB. RB undergo several rounds of replication inside the inclusion until they eventually undergo secondary differentiation back into EB (Abdelrahman and Belland 2005). The signal to induce EB to RB differentiation is not clearly defined, but it

may be due to a decrease in RB size after several rounds of replication (Lee et al. 2018; Chiarelli et al. 2020 Mar 14). EB then exit the host cell by one of two methods: host cell lysis or extrusion. The cell lysis pathway is an ordered process ending in cell death, similar to apoptosis. Lysis begins with the rupture of the chlamydial inclusion, followed by the rupture of other cellular compartments until the cell membrane is permeabilized and EB are released into the extracellular environment. Extrusion is an exocytosis-like pathway that leaves the host cell intact. During extrusion, the inclusion protrudes from the cell until it is released, packaged inside cellular plasma membrane with a thin layer of cytoplasm. Extrusions may then either burst open to release EB into the extracellular environment or be endocytosed by subsequent host cells (Hybiske and Stephens 2007; Zuck et al. 2016; Zuck et al. 2017).

Host Response to Chlamydia

The negative effects that *Chlamydia* inflicts on infected hosts are primarily driven by inflammation. For example, *C. trachomatis* serovars A-C causes trachoma, or chronic inflammation of the conjunctiva that can lead to scarring and blindness (Wright et al. 2008). Further, *C. trachomatis* serovars D-K causes genital infection, and if left untreated can cause inflammation in the genital tract leading to pelvic inflammatory disease, ectopic pregnancy, and infertility (Tsevat et al. 2017; Greydanus et al. 2021 Sep). Two primary hypotheses attempt to explain the cause of disease by *Chlamydia.* The immunological paradigm, proposed by Grayston, *et al.* in 1985, suggests that disease is driven by a chlamydial antigen that induces adaptive immune cells to drive inflammation (Grayston et al. 1985; Brunham and Rey-Ladino 2005). The cellular paradigm of chlamydial pathogenesis was proposed by Richard Stephens in 2003. This theory hypothesizes that *Chlamydia*-infected non-immune cells are a primary source of

inflammatory cytokines that recruit immune mediators that cause inflammation leading to disease (Stephens 2003; Murthy et al. 2018).

Non-Immune Cell Response

The mucosal epithelial cells lining the genital tract are the primary target of chlamydial infection as well as the host's first defense against infection. An *in vitro* study of *C. trachomatis*-infected HeLa cells showed that interleukin 8 (IL-8), a potent neutrophil chemoattractant, and granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine that stimulates myeloid cell production, are two of the pro-inflammatory factors released by epithelial cells within 20-24 hours of infection with *Chlamydia* (Rasmussen et al. 1997). This study, and other later studies using explanted fallopian tubes that supported its findings (Kelly et al. 2001; Hvid et al. 2007), suggests nonimmune epithelial cells are primarily responsible for the initiation of the inflammatory response to chlamydial infection that leads to scarring and other pathologies.

Innate Immune Response

Leukocytes such as neutrophils and macrophages are recruited to the site of chlamydial infection. They contribute to the killing of the pathogen as well as the release of proinflammatory cytokines and chemokines that contribute to further pathogenesis. Polymorphonuclear leukocytes (PMN) are white blood cells that include neutrophils, eosinophils, basophils, and mast cells. Sites of chlamydial infection are quickly infiltrated by PMNs *in vivo* (Rank et al. 2008). CXCL15, a PMN chemokine, is expressed as early as 3 hours after intracervical infection of mice with *C. muridarum*, and PMNs were found at the site of infection 12 hours after inoculation (Rank et al. 2010). PMNs can destroy *Chlamydia*-infected cells by phagocytosis and promote detachment of infected cells from the epithelial layer to reduce infection of nearby healthy cells

(Rank et al. 2008). Additionally, PMNs contribute to immunopathology, and the depletion of PMNs has been shown to reduce genital tract pathology in *Chlamydia*-infected mice pathology (Lijek et al. 2018).

Adaptive Immune Response

Several human and animal models of *Chlamydia* infection indicate that a type 1 helper T cell (Th1)-dominant cytokine profile results in the clearance of infection while a type 2 helper T cell (Th2)-dominant cytokine profile results in chlamydial persistence and disease progression (Stephens 2003). Interferon gamma (IFNγ)-producing Th1 cells are essential to bacterial clearance in *C. trachomatis*-infected mice (Ito and Lyons 1999; Gondek et al. 2009). In addition to IFNγ, tumor necrosis factor alpha (TNFα) is a cytokine released by T cells that has been shown to contribute to chlamydial clearance in humans (Jordan et al. 2017) and to genital tract pathology in mice and guinea pigs (Darville et al. 2000; Murthy et al. 2011).

Female Sex Hormones

Female sex hormones play pivotal roles in all biological processes, including the immune response to *Chlamydia* infection. Estrogen and progesterone are the primary female sex hormones, and their concentrations fluctuate as women of reproductive age progress through the menstrual cycle (Levin and Hammes 2011; Hoffman et al. 2012). Due to these fluctuations, studies of hormonal influence of biological processes must take into account the dominant hormones at various times of the menstrual cycle. The UN's World Contraceptive Use database reports that over 150 million women worldwide use oral hormonal contraceptives (2019). The addition of hormonal birth controls into the body influences estrogen and progesterone levels and adds another dimension to the study of these hormones in relation to infection and immunity.

The interactions of estrogen and progesterone with *Chlamydia* are reviewed in detail in chapter 2.

Estrogen

Estrogen is a steroid hormone found in several forms in the human body: estrone (E1), estradiol (E2), and estriol (E3) (Thomas and Potter 2013). E2 is the most potent and most common type of estrogen found in women of reproductive age and is the form given as hormone replacement therapy (Delgado and Lopez-Ojeda 2021 Apr 15). It is involved in innumerable biological processes. Of note, E2 facilitates the remodeling of female reproductive tissue and is an important regulator of immune function. Estrogen exerts its effects through two primary estrogen receptors (ER): estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) as well as a third receptor, G protein-coupled estrogen receptor (GPER-1) (formerly known as GPR30) (Eyster 2016). ERs are found in the cytoplasm and in the plasma membrane of cells to carry out nuclear and non-nuclear signaling, respectively (Katzenellenbogen et al. 2000; Madak-Erdogan et al. 2008). GPER-1 is reportedly involved in membrane-associated estrogen signaling (Prossnitz et al. 2008), although it does not compensate for estrogen signaling in the absence of primary ERs (Pedram et al. 2006a). A more detailed discussion of ERs can be found in Chapter 3.

Progesterone

Progesterone (P4) is also a steroid hormone involved in many biological processes. Like estrogen, it is important in the remodeling of female reproductive tissues and for immunological activity. P4 signaling is primarily mediated by the progesterone receptor (PGR). PGR is found in three forms, PGR-A, PGR-B, and PGR-C (Cable and Grider 2021 May 9), and is associated with nuclear as well as non-nuclear (membranous) pathways (Moussatche and Lyons 2012).

Mouse Models for Chlamydia Studies

Due to their relatively low cost and ease of use, mice are the predominant model used to study chlamydial infection *in vivo*. Mice can be infected with *C. trachomatis*, the primary agent of human genital *Chlamydia* infection. Additionally, *C. muridarum* is a chlamydial species that was first isolated from the lungs of mice (Nigg 1942) and causes ascending genital tract infection in mice that is similar to *C. trachomatis* genital infection in humans (Barron et al. 1981). *C. trachomatis* vaginal inoculation of mice does result in the ascension of genital tract like *C. muridarum* and requires a more concentrated inoculum as well as a more invasive inoculation method (transcervical inoculation) to establish infection (Ramsey et al. 2000).

C. trachomatis and *C. muridarum* infection of mice are both generally preceded by progesterone treatment in the form of depot medroxyprogesterone acetate (Depo-Provera or DMPA). Mice have a short, 4-5 day estrus cycle which results in the shedding of infected cells too rapidly for infection to be established. DMPA treatment halts the estrus cycle to allow for the establishment and subsequent study of chlamydial genital infection in mice (Tuffrey and Taylor-Robinson 1981). Therefore, the majority of murine chlamydial research has been done in progesterone-dominant conditions. However, humans are not in a state of constant progesterone dominance, which could be problematic for the application of DMPA-treated mouse studies to human chlamydial infection. This problem calls for development of a mouse model of chlamydial infection that does not require DMPA pretreatment. To overcome this issue, we used mice that are ovariectomized at an early age to stop the endogenous production of estrogen and progesterone. We implanted silastic capsules containing either E2, P4, or E2+P4 that delivered physiologically relevant doses of the hormones to the mice. We used an enzyme-linked immunosorbent assay (ELISA) to measure 17-β estradiol concentrations and liquid

chromatography coupled with mass spectrometry (LC-MS) to measure progesterone concentrations from serum collected from the mice. LC-MS methodology of hormone measurement is extensively reviewed in chapter 4.

CHAPTER 2. THE COMPLEXITY OF INTERACTIONS BETWEEN FEMALE SEX HORMONES AND *CHLAMYDIA TRACHOMATIS* INFECTIONS

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Keywords: *Chlamydia trachomatis,* Female sex hormones, Estrogen, Progesterone, Sexually transmitted infections

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Abstract

Purpose of Review: This review focuses specifically on the mechanisms by which female sex hormones, estrogen and progesterone, affect *Chlamydia trachomatis* infections in vivo and in vitro.

Recent Findings: Recent data support previous work indicating that estrogen enhances chlamydial development via multiple mechanisms. Progesterone negatively impacts Chlamydia infections also through multiple mechanisms, particularly by altering the immune response. Conflicting data exist regarding the effect of synthetic hormones, such as those found in hormonal contraceptives, on chlamydial infections.

Summary: Numerous studies over the years have indicated that female sex hormones affect *C. trachomatis* infection. However, we still do not have a clear understanding of how these hormones alter Chlamydia disease transmission and progression. The studies reviewed here indicate that there are many variables that determine the outcome of Chlamydia/hormone interactions, including: 1) the specific hormone, 2) hormone concentration, 3) cell type or area of the genital tract, 4) hormone responsiveness of cell lines, and 5) animal models.

1. *Introduction*

Concentrations of the female sex hormones (FSH), estrogen and progesterone naturally fluctuate with the menstrual cycle [1, 2]. Their presence in differing concentrations affects the physiology of the female genital tract (FGT), regulating ovulation, endometrial cell proliferation and maturation, and the immune response [1, 2]. Sex hormones even influence the composition of the vaginal microbiome [3, 4]. Because hormones are essential to the proper FGT function, the effects of estrogen and progesterone on the acquisition and progression of genital infections is an intriguing area of research. This review focuses specifically on the mechanisms by which endogenous and exogenous FSHs affect *Chlamydia trachomatis* infections.

Chlamydia trachomatis remains the world's predominant cause of bacterial sexually transmitted infection (STI) worldwide. Over one million chlamydial infections are reported to the Centers for Disease Control in the US each year. Although chlamydial infections are frequently reported in men, they are most commonly reported in young females [5]. Chlamydial infections initially present as cervicitis in women; however, many infections are asymptomatic. This is especially problematic as untreated chlamydial infections increase the patient's risk for developing pelvic inflammatory disease (PID), life-threatening ectopic pregnancy, and/or tubal infertility [6].

2. Influence of natural and synthetic FSH on Chlamydia infections in women

2.1. Complications of ascending chlamydial infections: salpingitis, and non-viable pregnancy

Few studies have directly examined the impact of estrogen or progesterone concentrations on transmission or progression of chlamydial infections in women. A 1986 study found that women infected with C. *trachomatis* or *Neisseria gonorrheae* were more likely to

develop salpingitis in the first seven days following menses, corresponding to the estrogen-dominant proliferative phase [7]. These observations suggest a correlation between estrogen and ascending chlamydial infections [7]. However, other studies have reported increased detection of Chlamydia from female patients during the progesterone-dominant secretory/luteal phase of the menstrual cycle, including a recent retrospective analysis of patient medical records [8, 9]. Unfortunately, these studies are often limited by small sample size. It is also hard to determine if hormones directly affected pathogen transmission by examining the time of detection, as this only reflects the time at which the infection became symptomatic, not necessarily when it was acquired. For instance, a cervical infection that is detected in the progesterone-dominant third week of the menstrual cycle may have been transmitted during the estrogen-dominant second week.

The impact of hormones on fertility and pregnancy outcomes in infected patients has also been explored. A study examining Chlamydia-associated oxidative stress in infertile women found increased levels of both oxidative stress markers and luteinizing hormone in Chlamydia-infected women compared to both Chlamydia positive and negative fertile women [10]. Non-viable pregnancies caused by spontaneous abortion or ectopic pregnancy are complications of chronic Chlamydia infections. There are multiple reasons that women experience spontaneous abortion, including an improper balance of hormones during pregnancy [11]. Decreased progesterone levels early during pregnancy can result in spontaneous abortion [12]. A prospective study measured estrogen and progesterone levels in Chlamydia-infected recurrent spontaneous aborters (RSA) during their first trimester. They found that these women had significantly increased estrogen and decreased progesterone levels compared to uninfected RSA and non-RSA control groups [12]. This study also demonstrated that Chlamydia-infected RSA

had increased expression of contractile prostaglandin receptors, which are regulated by hormone concentrations during pregnancy and are involved in initiation of labor [12]. While there could be other reasons for these observations, these data suggest that Chlamydia directly or indirectly cause a shift in estrogen and progesterone levels that can result in spontaneous abortion. LPS has been shown to increase the ratio of estrogen to progesterone in mice [13]. Thus, it is possible that Chlamydia infection alters the levels of hormones in the host leading to negative pregnancy outcomes.

2.2. Contraceptives and chlamydial risk

Globally, over 150 million of women rely on hormonal contraceptives (HC) for safe and effective birth control, many of whom are in the 14–29 age range that is at the highest risk for chlamydial infections [5, 14, 15]. Thus, it is likely that *C. trachomatis* will encounter an FGT environment that is regulated by synthetic hormones. Forcey, et al., demonstrated that HC use abolished the observed difference in detection of chlamydial DNA in the proliferative versus the secretory phases of the menstrual cycle, indicating that exposure to constant synthetic hormone levels alters the pattern of chlamydial infection compared to that in HC non-users [8]. Of the published studies examining the risk for chlamydial transmission with HC use, approximately 58% found a positive association [16]. Metaanalysis of 29 cross-sectional studies found a significant association between HC use and Chlamydia infection. Metaanlysis of 13 cross-sectional studies, which reported correction for some confounding variables, also found that HC use was associated with elevated chlamydial infection risk [17]. Three prospective studies noted a significant increase in chlamydial cervical infections with oral HC even after confounding variables, such as age, number of sexual partners and/or sexual behaviors, were taken into account [18–20]. Still, there are numerous studies that indicate no change in chlamydial infection

rate with HC use. A recent review of prospective studies published in the last 10 years found inconclusive evidence that Chlamydia infections were impacted by HC use [21].

A major hurdle to interpreting and comparing data from these clinical studies is that they are often inconsistent in design and analysis methods, leading to conflicting conclusions. Some studies do not account for confounding variables, such as the number of sexual partners or use of barrier contraception. Many of the studies also do not specify the type of HC used. This is important because there is a wide range of HC formulations and delivery mechanisms. Oral contraceptives are often combinations of a synthetic estrogen and progestin or a progestin-only mini pill [2, 22]. Long-acting progestin-only HC may also be injected or implanted in the body. Studies that record the type of HC used report varying conclusions. For example, one study found that the use of Depo-provera (DMPA) was associated with an increased risk of chlamydial infection, but found no increase in infection among women using oral contraceptives [23]. Conversely, another study found that combined oral contraceptives are a risk factor for ascending chlamydial infections where DMPA is not [24]. Levonorgestrel-intrauterine systems (LNG–IUS) release the synthetic progestin slowly over 3–5 years [25]. There have been concerns from obstetrician-gynecologists that LNG–IUS may result in increased risk of pelvic infection [26]. However, a study of US women showed that the risk of pelvic infection is not increased in the first few weeks after LNG–IUS placement [27]. Furthermore, STI testing and antibiotic treatment of Chlamydia-infected women at the time of LNG–IUS placement prevented pelvic infection [27]. These studies illustrate that HC use cannot be considered as a single experimental variable. To fully investigate the impact of HC on chlamydial infections, the type of HC must be known.

The primary HC mechanism of action is to prevent release of luteinizing and follicle-stimulating hormones from the pituitary gland, preventing ovulation. However, localized effects on endometrial and cervical cells are also observed [1, 2]. Though all synthetic progestins act as progesterone receptor agonists, interaction with mineralocorticoid, glucocorticoid, androgen receptors, and ERs has been documented, suggesting 'off-target' interactions could influence chlamydial development and/or pathogenesis in vivo [1, 2, 28–30]. Fichorova, et al., reported that immune biomarkers are differentially expressed in cervical samples from women that use oral HC versus DMPA and that infection with C. *trachomatis* can further alter the expression of inflammatory mediators under these hormonal conditions [14]. Therefore, HC may alter the immune response to sexually transmitted pathogens [31]. Several studies also speculate that HC-induced cervical ectopy is responsible for increased transmissibility of C. *trachomatis*; however, no molecular mechanism has been provided for these predictions [16]. It is likely that HC impact chlamydial infections in various ways depending upon the formulation and delivery mechanism employed. Thus, more investigation is needed to elucidate the specific effects of common synthetic hormones found in HC, as this information could inform physician recommendations when choosing HC for high-risk patients.

3. Animal models

Several animal models are used to study Chlamydia pathogenesis [32, 33]. Each of these models have advantages and disadvantages for examining the effects of hormones on chlamydial infections. Mice, due to their low cost, ease of use and extensive availability of reagents, are the most common animal model used in chlamydial research. However, mice have a short estrus cycle, lasting only 4–5 days, causing infected cells to be shed rapidly. To overcome this, mice are routinely treated with DMPA to prevent cycling [34]. Thus, most studies of chlamydial

pathogenesis in mice are performed under conditions of synthetic progesterone exposure. Interestingly, Pal et al. demonstrated that infecting mice with C. *trachomatis* in the progesterone-dominant phase yielded 20% more ascending infections than in the estrogen-dominant phase [35]. Progesterone pre-treatment is also required for establishing chlamydial infections in rats [36]. Therefore, these studies indicate that progesterone dominant conditions are favorable to chlamydial infections in rodents. Another caveat to rodent Chlamydia-infection models is that *C. muridarum* is often used, because it establishes a similar infection to humans when the animal is incoculated vaginally. To evaluate *C. trachomatis* infection in mice, the animals must be inoculated transcervically, bypassing natural ascension of the pathogen to the upper FGT from an initial cervical infection. Likewise, Göttingen minipigs must be transcervically inoculated with *C. trachomatis* during estrus to establish a lasting infection (>5 days) [37–39]. Intrauterine inoculation of minipigs with *C. trachomatis* during diestrus produces the longest duration of infection (≥10 days). The authors of these studies attributed increased IFNγ expression to faster clearance of *C. trachomatis* during estrus [37]. Conversely, studies evaluating *C. caviae* infection in guinea pigs found that pre-treatment with estrogen produced longer lasting infections with greater chlamydial shedding and pathology, while pre-treatment with progesterone had no effect on infection outcome [40–42]. Estrogen pretreatment is also essential for *C. trachomatis* serovars D and E infection in guinea pigs [43]. Unlike mice, the reproductive tract of guinea pigs has a more similar hormonal regulation to humans. Guinea pigs also have a longer estrus cycle (15–17 days), which eliminates the need for progesterone pre-treatment [32].

Non-human primate models serve as a physiologically relevant model for the study of *C. trachomatis* infections. The pig-tailed macaque has a menstrual cycle and vaginal microbiota that is very similar to humans [32]. Because they are naturally susceptible to *C. trachomatis*,

macaques do not require pre-treatment with progesterone [44]. Baboons also establish chlamydial infections with disease progression similar to humans [44, 45]. Interestingly, Eastman, et al., recently demonstrated that administration of Levonorgestrel via intrauterine system (LNG–IUS) increases *C. trachomatis* shedding, duration of infection and salpingitis in baboons [46]. These data suggest that chlamydial infections in non-human primates may be impacted by sex hormones, or at least synthetic progestins. While non-human primates are physiologically relevant models of human chlamydial infections, the effect of the endogenous hormonal environment on progression of chlamydial infection has not been investigated in these models.

4. In vitro studies

4.1. Potential interactions between Chlamydia and estrogen receptors

Estrogen receptors (ER) exist in two forms in the cell. First, there is the cytosolic receptor, which translocates to the nucleus to mediate gene transcription upon binding to the hormone ligand. Second, there are membrane ERs, which mediate non-genetic, rapid cellular signaling events in response to estrogen [47]. Membrane ERs and their membrane complex proteins have been associated with chlamydial entry into host cells [48–50]. Chlamydia enter host cells via clathrin-coated pits and caveolae, membrane structures known to contain ERs [48, 51–54]. Elegant studies by the Wryrick laboratory revealed that ERβ and protein disulfide isomerase (PDI), a member of the ER membrane complex, are associated with chlamydiae attached to the surface of host cells [50, 55]. Abromaitis and Stephens have shown that while PDI is not a receptor for attachment, its reductive functions are required for chlamydial entry into host cells [49]. Furthermore, inclusion development in cultured human endometrial cells was reduced by antibody blockage of membrane ERα/β or PDI, or by exposure to the ER antagonist,

tamoxifen, prior to C. *trachomatis* infection [50]. Additionally, inclusions observed in tamoxifen-exposed cultures were smaller than unexposed inclusions, suggesting that ERs are involved in inclusion development as well as EB attachment to the host cell [50].

In addition to aiding entry into host cells, there is evidence that chlamydiae may interact with ERs throughout the developmental cycle. CT441 is a protease secreted by *Chlamydia* with a PDZ domain that interferes with the NF–κB pathway by cleaving the host p65 protein. Multiple research groups have postulated that CT441 aids chlamydial host immune evasion by inhibiting NF–κB-mediated cytokine expression [56]. Steroid receptor RNA activator 1 (SRA1), a co-activator of ERα, is a functional RNA that is also translated to produce the protein SRAP1. CT441 directly interacts with, but does not cleave, SRAP1 in vitro and in vivo. CT441/SRAP1 binding prevents activation of ERα, reducing ERα localization to the nucleus and estrogen-mediated changes in gene transcription [57]. Overall, these studies suggest that ERs influence chlamydial infection in multiple ways. Most studies indicate that chlamydiae benefit from ERs via direct interactions or through hormone-modulated host cell signaling and gene expression. However, it is possible that in some circumstances or at certain times in development, *Chlamydia* benefit from shutting off ER activity and have evolved mechanisms to alter the cellular environment via interaction with hormone receptors.

4.2. Physiological environment: Hormone modulation of host cell signaling and gene expression

In the mid-1980s, Moorman et al. found that exposing human primary endometrial cell cultures to 10^{-7}M estrogen or a combination of 10^{-8}M estrogen/10^{-7}M progesterone decreased the percentage of cells infected with *C. trachomatis* [58]. Conversely, studies using HeLa cells or explanted human endometrial cells found that exposure to 10^{-10}M estrogen increased attachment of chlamydiae to host cells, while exposure to 10^{-8}M estrogen or a combination of

estrogen/progesterone had no effect or decreased infection with *Chlamydia trachomatis* [59, 60]. Subsequent studies demonstrated that *C. suis* S45 infection in swine genital epithelial cells behaved similarly to *C. trachomatis* Serovar E infection in explanted human endometrial epithelial cells. They confirmed that cells harvested during the estrogen-dominant phase of the menstrual cycle were more susceptible to chlamydial infection than cells harvested during the progesterone-dominate phase [61]. Moreover, Guseva, et al., noted that swine genital epithelial cells harvested during a particular phase of the menstrual cycle could not be reprogramed by exogenous hormone exposure [61]. These early data suggested that i) hormone concentration is a very important determinant of how estrogen impacts chlamydial infection, and ii) the varying physiological effects of hormones on different potential host cells may contribute to the enhancing or inhibitory nature of estrogen and progesterone on chlamydial infections.

Recently, we studied the effects of estrogen and progesterone on chlamydial infection using a co-culture model of immortalized human endometrial epithelial cells (Ishikawa, IK) and stromal (SHT–290) cells. This model more closely mimics an *in vivo* environment than previous studies using exogenous hormone supplementation of genital epithelial monocultures. Hormonal regulation of the endometrial epithelial cell cycle relies on a combination of direct interaction of hormones with epithelial cell receptors as well as paracrine signals released by underlying stromal cells in response to hormonal stimulation [62]. C. *trachomatis*-infected IK/SHT–290 co-cultures exposed to 10^{-8}M estrogen produced significantly more inclusions and progeny *Chlamydia* compared to hormone-free controls [50, 63]. We also exposed IK/SHT–290 co-cultures to progesterone dominant conditions mimicking those in the secretory phase. To do this, co-cultures were primed with 10^{-8}M estrogen prior to adding a combination of 10^{-9}M estrogen/10^{-7}M progesterone. When infected under progesterone dominant conditions,

chlamydial infection was decreased compared to estrogen-exposed samples [63]. Interestingly, the hormone-mediated positive or negative effects on chlamydial infection in IK cells were only observed when the stromal cells were present, suggesting that secreted stromal cell effectors are important components of the observed changes in chlamydial infection [50, 63]. Stromal cells release a variety of cell signaling molecules that, along with direct estrogen and/or progesterone signaling, regulate proliferation and maturation of endometrial epithelial cells. Notably, estrogen increased phosphorylation of ERK, a member of the MAPK pathway, in IK cells during IK/SHT–290 co-culture, but not in IK cultures alone. Cytokine expression in the presence of estrogen was also reduced in IK/SHT–290 and HEC-1B/SHT–290 co-cultures [50]. These data suggest that paracrine stromal cell signaling influences the impact of hormones on chlamydial infection and may act through regulation of host cell signaling pathways.

Studies have demonstrated that human FSHs also mediate gene expression of a significant proportion of chlamydial genes. The expression of approximately 25% of the chlamydial transcriptome was altered 2-fold or greater in response to estrogen and/or progesterone exposure in infected ECC–1 cells. Specifically, estrogen-exposure downregulated chlamydial genes involved in fatty acid and nucleotide biosynthesis. Additionally, estrogen upregulated genes involved in chlamydial persistence, suggesting that estrogen may promote the chlamydial stress response in ECC–1 cells [64]. This is an interesting finding given that other human cell culture models of estrogen exposure demonstrate that estrogen promotes chlamydial infection and progeny production [40, 50, 59–61]. Progesterone did not affect expression of the same chlamydial genes as estrogen in infected ECC–1 cells, but did alter accumulation of a substantial number of chlamydial transcripts, including those involved in the TCA cycle,

glycolysis, and carbohydrate and amino acid metabolism. In all, progesterone upregulated expression of 85 and down-regulated expression of 135 chlamydial genes [64].

As with C. *trachomatis*/ER interactions, these studies collectively confirm that hormone modulation of the host cell environment can have different effects on chlamydial infection depending on several factors, including cell line or model system, primary vs immortalized cells, and concentration of hormones. This make data interpretation and comparisons between studies difficult. However, regardless of the experimental variables, the results from these studies support the conclusion that sex hormones impact in vitro chlamydial infection in multiple ways.

5. Immune Response

Immunity in the FGT is uniquely designed so that it protects the genital tract from infection, while allowing a fetus to develop during pregnancy. Estrogen and progesterone's role in regulating the FGT immune system has been extensively reviewed by others [31, 65–67]. It is also hypothesized that HC influence STIs by altering the host's immune response [3]. Several studies indicate that sex hormones alter the expression of immune factors or immune cell responses during chlamydial infection. Like previous studies, progesterone exposure significantly reduced chlamydial infection in ECC–1 cells, as measured by decreased accumulation of *C. trachomatis* DNA. Cytokine and chemokine expression was increased in progesterone-exposed *C. trachomatis*-infected ECC–1 cultures compared to estrogen-exposed cultures, suggesting that progesterone's inhibitory effect on *Chlamydia* is immune-mediated [68]. Agrawal, et al., showed that *C. trachomatis*-infected cells pre-exposed to estrogen had reduced TLR4 expression and Th1–associated cytokines compared to infected cells not exposed to estrogen. The anti-inflammatory cytokine IL–10, however, was significantly increased in infected, estrogen-exposed cells, suggesting that estrogen may promote a less effective Th2

immune response to *C. trachomatis* infections [69]. Conversely, other studies suggest that estrogen-mediated immune factors protect against chlamydial infections. Interferon ε (IFNε) is a type 1 interferon expressed in the FGT whose function is not well understood. Its expression varies throughout the menstrual cycle with the highest expression occurring during estrogen-dominant conditions. *C. muridarum*-infected IFNε $^{-/-}$ mice had more severe infections and bacterial shedding than wild type mice. These data suggest that estrogen-stimulated expression of IFNε may promote clearance of C. *muridarum* from mice [70]

Hormones also affect antibody responses during chlamydial infection. The role of secretory IgA in clearance of chlamydial infection has been debated in several studies. Armitage, et al. showed that murine expression of the polymeric immunoglobulin receptor plgR, which is essential for transcytosis of IgA in the FGT, is increased during estrus, whereas DMPA decreased plgR expression and IgA accumulation in the tissues. Although *C. muridarum* infection increased plgR expression in DMPA pre-treated mice, the availability of IgA at the time of inoculation was lower than it would have been had the mice been infected during estrus. These data raise the possibility that DMPA pre-treatment masks the contributions of IgA to infection clearance in the mouse model [71].

While clinical studies show that LNG–IUS use is not associated with increased pelvic infection incidence [27], *in vitro* studies show that LNG affects immune cells during *Chlamydia* infection. Primary human dendritic cells (DCs) exposed to LNG had decreased expression of CD80, CD86 and CD40 and were inhibited in their ability to activate naïve T cells in vitro. Additionally, incubating LNG–exposed DCs with inactivated *C. trachomatis* significantly reduced the DC CD40 expression, suggesting that LNG alone as well as LNG in the presence of *Chlamydia* alters expression of immune-associated factors [72]. Mice

implanted with LNG pellets and infected intra-nasally with *C. trachomatis* had significantly fewer CD40–expressing DCs in their cervical lymph nodes than mice that received placebo pellets. LNG-treated mice were also not able to clear infection from the lungs compared to placebo-treated mice. Interestingly, clearance of infection by mice without CD4+ T cells was similar to that of LNG-treated mice [72]. Overall, these studies show that LNG reduces the ability of DCs to mature and activate T cells *in vitro* and *in vivo*, both in the presence and absence of *C. trachomatis*. *C. trachomatis*-infected baboons implanted with a LNG–IUS responded to infection with a Th2 response, while animals without LNG–IUS responded with a cell- mediated Th1 response. Baboons with a LNG–IUS shed greater numbers of *C. trachomatis* for a longer period of time and were more likely to develop PID than animals without the LNG–IUS. These results indicate that LNG–IUS contraception increased the risk of PID by hormonal-modulation of the immune response [46].

Overall, these studies highlight the numerous effects of natural and synthetic hormones on immune function. It is likely that hormone-induced changes in the immune response impacts chlamydial infections. Indeed, hormonal modulation of the immune system is hypothesized to alter transmission and disease progression for other STI pathogens, notably HIV [19, 73]. When considering these studies, it is important to note that different areas of the FGT (uterus, Fallopian tubes, cervix) do not respond to hormone-stimulated signals in the same manner. For example, cytotoxic T lymphocyte activity in the uterus and Fallopian tubes is suppressed during the secretory phase, where as others found no difference in cytotoxic T lymphocyte activity in the cervix between the proliferative secretory phases [67]. Similar differences have also been observed for other immune functions, like secretory IgA and chemokine receptor expression

[67]. Therefore, it is possible that hormones impact *C. trachomatis* infection differently at varying sites within the FGT by tissue specific changes in the immune response.

6. Future Directions and Conclusions

While the effects of estrogen and progesterone on *Chlamydia* infection have been investigated for decades, we still do not have a clear understanding of how these hormones affect *Chlamydia* disease transmission and progression. This is not surprising given the complexity of hormone signaling in the FGT. The data presented thus far indicate that FSHs affect *Chlamydia* via multiple mechanisms. The estrogen receptor may directly or indirectly aid EB host cell entry [48, 50]. Endogenous estrogen and progesterone often have opposing effects on chlamydial infection by altering the physiological environment through regulation of host-cell signaling pathways, paracrine stromal cell signals, chlamydial gene transcription and the immune response [41–43, 50, 60, 61, 63, 74]. Synthetic hormones found in HCs also impact chlamydial infection, but not necessarily in the same manner as endogenous hormones. Additionally, the published studies reviewed here indicate that there are many variables that determine the outcome of *Chlamydia*/hormone interactions, including: 1) the specific hormone used (natural or synthetic compounds), 2) hormone concentration, 3) cell type or area of the genital tract examined, 4) hormone responsiveness of tissue culture cell lines, and 5) the animal model used. Future research should be targeted to determine the exact mechanisms by which natural and synthetic forms of FSHs influence chlamydial development, keeping the stated experimental variables in mind.

To date, most studies examining hormonal effects on *Chlamydia* focused on hormone-host cell/immune cell-pathogen interactions. However, it is important to remember that sex hormones influence the entire body, not just genital tract cells. Hormones affect the availability

of nutrients in the environment. For example, estrogen regulates iron levels in the body, a key nutrient for C. *trachomatis* [75, 76]. Microbial endocrinology is a relatively new but emerging field of research because of the widespread involvement of the microbiome with the host. It has been suggested that bacteria interact with eukaryotic hosts through an interplay of quorum sensing molecules and host hormones [77]. Hormonal fluctuations during the menstrual cycle may alter the vaginal microbiome composition, which is known to influence the outcome of vaginal infections through direct (Ex: maintenance of low vaginal pH) and indirect mechanisms (Ex: modulation of immune responses) [78]. The role of the host vaginal microbiota is an understudied factor in the interactions with FSHs and *Chlamydia.* Studies have suggested that *C. trachomatis* infection is associated with altered diversity in the vaginal microbiome, as reviewed by Molenaar, et al. and others [73, 79]. In baboons, *C. trachomatis* infection in the presence of the LNG–IUS decreased the total number of microbiome-associated bacteria, but not the diversity of the vaginal microbiome [78]. Therefore, it is possible that hormones indirectly influence *Chlamydia* transmission by altering the vaginal microbiota.

Another aspect of hormone-*Chlamydia* studies that is worth further investigation is the relevance of the most common animal models used for *Chlamydia* research. Because rodents require progesterone pre-treatment for the establishment of chlamydial infection, the majority of *in vivo* studies on *Chlamydia* are under the influence of DMPA. Although these studies have provided a wealth of knowledge about chlamydia infections, there is evidence that DMPA influences the outcome of mouse infection studies. As noted above, DMPA reversed estrogen-stimulated expression of plgR, possibly masking the value of IgA in clearing chlamydial infections [71]. Kaushic, et al., demonstrated that when mice were treated with DMPA prior to vaccination with an attenuated strain of Herpes Simplex Virus (HSV) and subsequent challenge,

none of the animals were protected. However, when mice were exposed to natural progesterone before vaccination, they were protected from lethal HSV challenge [80]. Thus, it is worth determining if DMPA treatment in mice skews the progression and outcome of chlamydial infection, particularly in the case of vaccine research.

Although numerous studies have investigated the effects of FSHs on chlamydial infection, the exact mechanisms of these interactions have not completely been elucidated. Future research is crucial to increase our understanding of the roles of sex hormones in the entry, establishment, and pathogenesis of *Chlamydia*. A better understanding of these interactions will help researchers and medical professionals improve treatments, contraceptive recommendations and develop vaccines against *Chlamydia*.

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CHAPTER 3. ESTROGEN AND ESTROGEN RECEPTORS INFLUENCE *CHLAMYDIA MURIDARUM* INFECTION IN MICE

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Abstract

Genital *Chlamydia* is the most common bacterial sexually transmitted infection in the United States and worldwide, causing severe sequelae in women such as pelvic inflammatory disease and irreversible tubal infertility. Previous studies indicate that the progression of chlamydial infection is influenced by various factors, including the female sex hormones estrogen and progesterone. Sex hormone levels naturally fluctuate in women throughout their menstrual cycle. Varying concentrations of estrogen and progesterone may impact the progression of chlamydial infection and the host's immune response to *Chlamydia*. Estrogen signals through estrogen receptors (ERs), ERα and ERβ. These receptors are similar in structure and function, but are differentially expressed in tissues throughout the body, including the genital tract and on cells of the immune system. In this study, we used ovariectomized (OVT) Balb/c mice to investigate the impact of estrogen (E2), progesterone (P4), or a combination of E2/P4 on the progression of and immune response to *C. muridarum* infection. Additionally, we used ERα and ERβ knockout C57/BL6 mice to determine the how ERs affect chlamydial infection and the resulting immune response. We found that estrogen completely protects mice from chlamydial infection, even in the presence of progesterone, and that the absence of ERα, but not ERβ, significantly affects the timing of *C. muridarum* infection. At day 9 post infection, flow cytometry showed that ERαKO mice had more T cells present at and targeted RNA sequencing revealed increased expression of CD4 and FOXP3, suggesting that ERαKO mice had increased numbers of regulatory T cells compared to ERβKO and WT mice. Through these studies, we postulate that ERα influences the immune response to chlamydial infection through the regulation of the T cell response.

Introduction

Chlamydiae are obligate intracellular bacteria that cause mucosal infections in vertebrates including humans, other mammals, and birds. Primary sites of infection include the conjunctiva, the respiratory tract, and the genital tract (Bachmann et al. 2014). Genital *Chlamydia* infections in humans are caused by *Chlamydia trachomatis*, the most commonly reported bacterial sexually transmitted pathogen in the United States (US) and worldwide. Reported cases of genital *Chlamydia* infections are rising with a 2.9% increase in reported cased in the US from 2017-2018 (Kreisel et al. 2021)*C. trachomatis* exists in two developmental forms as it progresses through its biphasic life cycle: infectious, non-replicative elementary bodies (EB) and non-infectious, replicative reticulate bodies (RB) (Moulder 1991). As chlamydial infection progresses in females, it ascends the genital tract, invading the uterus and fallopian tubes. Left untreated, this can result in severe sequelae such as pelvic inflammatory disease, ectopic pregnancy, and irreversible tubal infertility (Paavonen 1999).

Several studies indicate that estrogen and its receptors are involved in the establishment and progression of chlamydial infection. Estrogen is a female sex steroid hormone that primarily signals through two estrogen receptors (ERs): estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), as well as a G protein-coupled estrogen receptor, GPER-1 (formerly known as GPR30) (Eyster 2016). Cytoplasmic ERs alter gene transcription via two mechanisms when activated by estrogen. In the classical mechanism, estrogen binds the ER causing translocation to the nucleus and activation of gene transcription via direct binding to DNA (Katzenellenbogen et al. 2000). In the tethered mechanism, estrogen-stimulated ERs form complexes with DNA-bound transcription factors to modulate the activity of transcription factors (Jakacka et al. 2001; Heldring et al. 2011) In addition to these nuclear signaling mechanisms, classical ERs (ERα and

ERβ) act in a non-nuclear fashion by stimulating protein kinases such as c-Src and MEK (Pedram et al. 2006b; Madak-Erdogan et al. 2008). Membrane ERs and nuclear ERs are derived from the same mRNA transcript (Razandi et al. 1999; Chambliss et al. 2002). The amount and identity of ERs (α or β) found at the plasma membrane is dependent on cell type, but it is estimated that 5-10% of ERs are located in the plasma membrane (Levin 2009). Estrogen signaling via membrane ERs triggers cAMP and ERK activity to stimulate non-genomic effects (Razandi et al. 2000; Song et al. 2002; Pedram et al. 2006b). GPR30 is reported to mediate membrane-associated estrogen signaling (Prossnitz et al. 2008), however, some reports contradict this observation, noting that the absence of GPR30 does not affect estrogen signaling and GPR30 does not compensate for estrogen signaling in the absence of ERs (Pedram et al. 2006b).

The effects of estrogen on chlamydial disease progression are likely multifactoral in nature. Estrogen exposure has been shown to affect the expression of toll-like receptors (TLR) as well as chemokine and cytokine production (Agrawal et al. 2013; Fung et al. 2013). EB interact with the surface of epithelial host cells through protein disulfide isomerase, a component of the membrane estrogen receptor complex (Davis et al. 2002). These studies indicate that research must evaluate the contribution of both chlamydial biology and the host immune response when determining the effects of ERs on chlamydial pathogenesis.

As human females progress through a typical 28-day menstrual cycle (Caligioni 2009), estrogen and progesterone levels naturally rise and fall. As estrogen levels rise during the follicular phase of the menstrual cycle, both the endometrium and vaginal epithelium thickens and cervical mucus increases. Following ovulation, estrogen levels fall and progesterone becomes the predominant hormone as the uterus prepares for either implantation or menstruation

At the end of the secretory phase, progesterone levels decline, causing the endometrial epithelial lining to be shed during menses. Varying levels of estrogen affect the expression of cytokines, chemokines, pattern recognition receptors, and other immune components (Wira et al. 2015). The estrous cycle in mice is similar to the reproductive cycle in humans, though it is much shorter at 4-5 days in length (Caligioni 2009). During the murine estrous cycle, estrogen concentrations rise during proestrus, remain elevated during estrus, fall during metestrus, and begin to increase again during diestrus (Caligioni 2009). The concentration of progesterone in mice is low during proestrus and estrus, begins to rise during metestrus, and peaks during diestrus (Walmer et al. 1992).

Our lab previously used the estrogen-responsive endometrial cell line, Ishikawa, co-cultured with the stromal cell line SHT-290, to show that 17-β estradiol treatment prior to infection with *C. trachomatis* resulted in an increase in chlamydial inclusion development and infectious EB production. Additionally, antibody blockage of ERα and/or ERβ decreased chlamydial infectivity in both Ishikawa and Hec-1b cell lines (Hall et al. 2011). Our prior data, along with the understanding that estrogen/ER signaling modulates the immune response suggests that ERα and/or ERβ are involved in the host's response chlamydial infection. While the IK/SHT-290 co-culture model is useful for examining the effects of hormone-induced, stromal/epithelial cell communication on chlamydial infection, it does not recapitulate the *in vivo* setting with a functioning immune system. Thus, the current study aims to elucidate the complex network of interactions between estrogen/estrogen receptor activity, chlamydial infection, and the immune response using a *C. muridarum* infection model.

Materials/Methods

Chlamydia and tissue culture cell lines

Chlamydia muridarum strain *Nigg* was cultured in HeLa 229 cells grown on Cytodex microcarrier beads. EB were harvested by centrifugation and stored in 2SPG [0.02 M phosphate buffer, 0.2 M sucrose, 5mM glutamine (pH 7.2)] at -80°C. The human cervical cell line HeLa 229 was maintained in Minimal Essential Medium (Gibco) with 10% fetal calf serum (FBS) and 0.01% gentamycin.

Mouse strains

To investigate the effects of estrogen and progesterone on *C. muridarum* infection in an experimentally controllable animal model, ovariectomized (OVT) or sham-OVT female Balb/c mice were ordered from the Jackson Laboratory (Bar Harbor, ME). Surgery was performed at 5 weeks old, and mice were delivered at 6 weeks old. Upon arrival, vaginal washes were performed with 20ul 1X PBS to ensure the mice were not experiencing an estrous cycle. Four genotypes of 6–8-week-old C57/BL6 mice were ordered from the Jackson Laboratory (Bar Harbor, ME): B6N(Cg)-*Esr1*$^{tm4.2Ksk}$/J (ERαKO), C57BL/6NJ (ERαWT), B6.129P2-*Esr2*^{tm1Unc}/J (ERβKO) and C57BL/6J (ERβWT) to investigate the specific effects of ERα and ERβ signaling on *C. muridarum* infection.

All mice were housed 4-5 per cage in a facility with a 12:12 light/dark cycle and provided with food and water *ad libitum*, according to our vertebrate animal protocol as approved by the ETSU University Committee on Animal Care, accredited through the Assessment and Accreditation of Laboratory Animal Care.

Making and insertion of silastic capsules

Mice were exposed to estrogen or progesterone via a surgically implanted silastic capsule as described by Ingberg, *et al.* (Ingberg et al. 2012) and Strom, *et al.* (Ström et al. 2012). Solutions of 36 ug/ml 17-β estradiol (E2), 50 mg/ml progesterone (P4), and 36 ug/ml 17-β estradiol + 50 mg/ml progesterone (E2/P4) were prepared in sesame oil (SO). Two cm lengths of silastic tubing were filled with the appropriate solution (E2, P4, E2/P4, or SO alone) and the ends were plugged with 3mm wooden applicator sticks. Capsules were placed in the remaining hormone solution and left on a rocker at 37°C for 2 days to equilibrate.

OVT or sham-OVT mice were anesthetized with isofluorane. A 2x2 cm area between the mouse's shoulders was shaved and cleaned with iodine. An incision was made, and a subcutaneous pocket was bluntly dissected to make space for the capsule. The capsule was inserted, and the incision was closed with 1-2 staples. Mice were monitored for two days post-surgery to confirm the incision was healing and the capsule was retained. One-week post-surgery, the staples were removed under isofluorane anesthesia.

C. muridarum infection and titer of progeny EB

Mice were vaginally infected with 500 IFU of *C. muridarum* in 2SPG eight days post hormone capsule insertion (OVT mice) or one week after depo-medroxyprogesterone acetate (DMPA, 2mg/kg) treatment (non-OVT mice). Every three days post infection (pi) mice were vaginally swabbed with a calcium alginate tipped swab, which was stored in a tube with glass beads at -80°C. Hela 229 monolayers were infected in duplicate with a dilution of the mouse vaginal swab samples by centrifugation. Infected monolayers were incubated 24 hours before methanol fixation. Inclusions in the fixed samples were stained with anti-LPS stain (Biorad) and

viewed using a Ziess Axiovert fluorescent microscope. The number of inclusion forming units (IFU) were counted on each coverslip and IFU per swab sample was calculated to create a 21 day EB shed curve for each mouse (Phillips Campbell et al. 2012).

Serum collection for hormone measurements

Blood was collected via cheek bleed into a 1.5 mL tube and left at room temperature for 30 minutes, then centrifuged at 2000xg for 10 minutes at 4°C. Serum was removed and stored at -80°C until testing by ELISA for 17- ERβ estradiol or LC-MS/MS for progesterone.

ELISA determination of estrogen from serum

Estrogen concentration in serum samples was measured using the Calbiotech E2 ELISA (ES180) according to the manufacturer's instructions and absorption was read using a Turner Modulus plate reader.

LC-MS/MS determination of progesterone from serum

For each progesterone measurement, 100 mL of serum was vortexed with 10 mL of internal standard (100 ng/mL d9 labeled progesterone Sigma-Aldrich, St. Louis, MO, USA), then combined with 100 mL LC-MS grade water. The diluted sample was then deposited into a Biotage ISOLUTE SLE+ 400 µL 96-Well Plate and a vacuum pulse was applied to load the sample. Samples were allowed to interact with SLE+ packing for five minutes before elution with ethyl acetate (2x aliquots of 400 mL each). Final elution was achieved using vacuum and the resulting eluent was evaporated to dryness under nitrogen at 37°C using a TurboVap LV

(Biotage, Uppsala, Sweden). The residue was reconstituted in 80 mL of LC-MS grade acetonitrile and then filtered using a 0.22μm filter tube (CoStar Group, Washington DC, USA). The filtered sample was then subject to LC-MS/MS analysis using a Shimadzu LCMS-IT-TOF system (Shimadzu Scientific, Kyoto, Japan). Separation occurred with a UCT C18 column (100 x 2.1 mm, 1.8-micron particle size) maintained at 50°C. The HPLC program included a gradient elution with 1mM ammonium fluoride in water (A) and acetonitrile (B) with a 30% B to 100% B ramp over 10 minutes. The mass spectrometer was maintained in +ESI mode using nitrogen as a nebulizing gas (1.5 L/min). Quantification was achieved using the direct MS channel for progesterone and d9-progesterone, m/z 315.01 and m/z 324.12, respectively. On each day of sample analysis, a five-point calibration curve (5, 10, 20, 50, and 100 ng/mL progesterone, Sigma-Aldrich, St. Louis, MO, USA) was prepared by spiking blank serum with progesterone stock solution to achieve the desired concentrations. Then the progesterone spiked samples were subject to the same internal standard concentration and SLE+ extraction prior to analysis. Calibration curves were created by plotting peak area ratio (progesterone: d9-progesterone) versus spiked progesterone concentration and a best-fit line was generated (Supplemental Figure 2). The best-fit line was used to calculate progesterone concentration in study sample using their peak area ratios.

Tissue collection

Mice were sacrificed on day 9 or day 21 pi. Genital tracts were observed for gross pathology *in situ.* The genital tracts were scored for redness and swelling as described in Table 3.1. Photos were taken to create a scoring reference sheet so that genital tract pathology was consistently scored between groups and experiments Supplemental Figure 3.1. Afterwards, the tissues were excised from the animals and separated into 3 parts: ovaries, uterine horns, and

cervix. Tissues for flow cytometry and RNA analyses were minced and placed in 1mg/ml collagenase in Gibco Hank's balanced salt solution (HBSS). After incubation in collagenase for 30 minutes at 37°C, tissues were homogenized with a pestle. A portion (100ul) of each sample was then mixed with RLT buffer (Qiagen RNeasy Kit) to be used for RNA analysis. The remainder of each sample was filtered, rinsed with MEM followed by 0.5 M EDTA. Single cells were collected by centrifugation and resuspended in MEM for flow cytometry analysis. This tissue collection method was derived from Nagarajan et al (Nagarajan et al. 2012).

Table 3.1: Pathology Score Criteria

Score	Explanation
0	No redness, swelling, or other pathology present
1	Swollen with no color OR pink with no swelling
2	Pink and mild swelling
3	Red with or without swelling
4	Dark red and severe swelling
5	Dark red, severe swelling, and hydrosalpinx or cyst(s)

Flow cytometry analysis

Tissues were collected as described above. Cells from the ovary, uterine horn, and cervix from each mouse were combined to ensure an ample number of cells for flow cytometric measurement. A blocking buffer including 5% BSA, rabbit serum, EDTA, and Fc blocking antibody (CD16/CD32 Rat anti-Mouse; BD Biosciences 553142) was added to each sample to

block leukocyte staining. Cells were stained overnight with the following antibodies at 4°C: Pacific Blue™ Hamster Anti-Mouse CD3e (T cells), PE Rat Anti-Mouse CD19 (B cells), Rat Anti-Mouse Ly-6B.2 Alloantigen: FITC (PMNs), PE-Cy™7 Rat Anti-Cd11b (granulocytes), PerCP-Cy™5.5 Rat Anti-Mouse Ly-6G and Ly-6C (GR-1) (granulocytes), and AF 647 Rat Anti-Mouse F4/80 (monocytes/macrophages), or their isotype control antibodies. All antibodies were ordered from BD Biosciences, with the exception of Ly-6B.2, which was ordered from BioRad. The following day, samples were washed with stain buffer, fixed in 4% paraformaldehyde, suspended in stain buffer, and analyzed using a BD Fortessa flow cytometer with FACSDiva software. Murine blood was collected from a cull mouse via cardiac puncture and stained with all 6 antibodies for use as a positive control for the detection of positively stained cell populations. The fully stained blood sample was used for the gating of lymphocyte and myeloid cell populations based on forward versus side scatter plots and positive antibody staining. Lymphocytes were gated based on positive CD3e versus CD19 staining and myeloid cells were gated based on positive Ly-6G and Ly-6C versus Ly-6B.2, and CD11b versus F4/80 staining.

TruSeq targeted RNA expression

RNA was collected as described above and processed as detailed in the TruSeq targeted RNA expression guide (Illumina). The quality of RNA was checked using an Agilent Technologies 2100 Bioanalyzer with an Agilent RNA 6000 Nano Kit. cDNA was synthesized from each RNA sample, then an oligo pool with up- and downstream primers specific to our target genes were hybridized the cDNA on a thermocycler set to 70°C for 5 minutes, 68°C for 1 minute, 65°C for 2.5 minutes, 60°C for 2.5 minutes, then the temperature was decreased from 55°C to 30°C in 5° increments for 4 minutes each. Samples were washed to remove excess

oligos, and then a DNA polymerase was added to extend oligos through the target regions. The libraries were then amplified, then index adapters were added. The libraries were then cleaned up and pooled to prepare for quantification. An Agilent DNA 1000 kit was used to determine the concentration of the pooled library. The library was analyzed by the University of Tennessee Genomics Core Facility (Knoxville, TN) on an Illumina Miseq. Data was analyzed using Qiagen's CLC Genomics Workbench following the RNAseq workflow.

Statistics

Unless otherwise stated the data presented represent data collected from three independent experiments with 4-8 mice/group. Data was analyzed using Microsoft Excel and GraphPad Prism software. Bacterial shedding between mouse groups was compared using two-way ANOVA and independent 2-sample t-tests a where appropriate. Immune cell populations measured by flow cytometry were reported as percentages of total cells measured, and compared using a one-way ANOVA and 2-sample t-tests. Qiagen's CLCBio Genomics Workbench was used to analyze RNAseq data. The differential expression for RNASeq tool using trimmed mean of M values (TMM) normalization was used to compare gene expression among groups and groups with statistically significant fold changes were reported. In all analyses, significance was defined as $p<0.05$.

Results

17-β Estradiol protects mice from C. muridarum infection

Since the murine model is the most commonly used whole animal model in chlamydial research, we chose to use an OVT mouse model to examine the effects of physiologically relevant concentrations of female sex hormones on chlamydial infection. Silastic capsules containing either sesame oil (SO) only (HF), 17-β estradiol in sesame oil (E2), progesterone in SO (P4), or a combination of 17-β estradiol and progesterone in SO (E2/P4) were inserted subcutaneously into OVT Balb/c mice. Mice with a sham ovariectomy and sham capsule surgery were also included in the study as controls. To ensure the hormone levels were within physiological range, concentrations of 17-β estradiol were measured by ELISA and progesterone concentrations were measured by LC MS/MS from serum collected the day before infection (day -1) and at day 21pi (Figure 3.1)

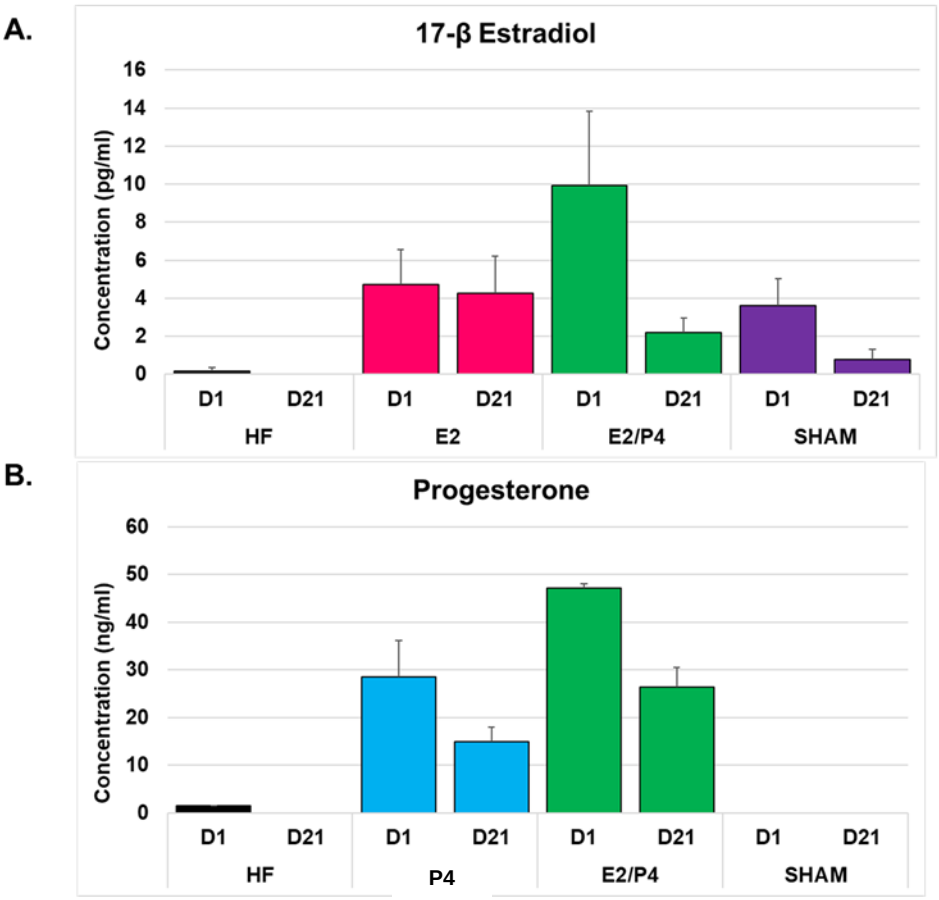

Figure 3.1: Measurement of E2 and P4 from mouse serum A. ELISA was used to measure 17-β estradiol concentrations from mouse serum. B. LC MS/MS was used to measure progesterone concentrations from murine serum. **E2:** 17-β estradiol, **P4:** progesterone, **E2/P4:** combination 17-β estradiol and progesterone, **HF:** hormone free, **sham**: sham-ovariectomized and sham-capsule insertion

At day 0 HF, E2, P4, E2/P4 or sham mice were mock or *C. muridarum* vaginally infected. Vaginal EB shedding was monitored for 21 days following infection. There was no significant difference in EB shedding between sham, HF, or P4 exposed mice. On the contrary, estrogen exposure, alone (E2) or in combination with progesterone (E2/P4) abolished EB shedding indicating that 17-β estradiol protected the mice from establishing chlamydial infection (Figure 3.2).

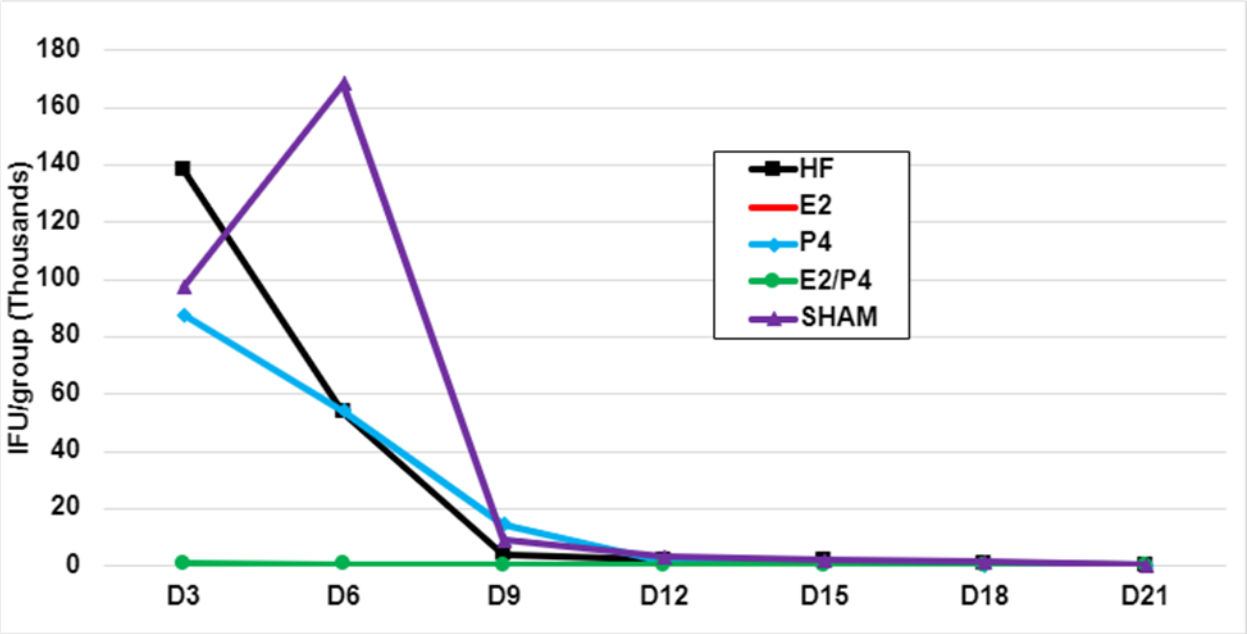

Figure 3.2: EB shedding from ovariectomized mice treated with hormone-filled capsules. **E2:** 17-β estradiol, **P4:** progesterone, **E2/P4:** combination 17-β estradiol and progesterone, **HF:** hormone free, **sham**: sham-ovariectomized and sham-capsule insertion, **IFU:** inclusion forming units

Estrogen influences the T cell response to C. muridarum

Given that studies have shown that hormones influence immune cell function and signaling, we sought to determine if the observed changes in EB shedding in OVT mice are due to hormonal influences on the immune response. A subset of hormone-exposed OVT and sham mice were sacrificed on day 10pi to determine the immune cell phenotype present in mock or *C. muridarum* (Cm)-infected genital tracts at that time (Figure 3.3). Day 10pi was chosen because EB shedding had decreased for all groups by this day and previous studies have shown that *C. muridarum* EB shedding typically peaks by day 6pi and has begun to decrease by day 9pi. Flow cytometry showed that of the immune cells measured (T cells, B cells, PMNs, and monocytes/macrophages), the T cell population was the most altered depending on hormonal condition. There were significantly fewer T cells present in E2 and E2/P4 groups compared to

sham, HF, and P4-treated groups. These data are unsurprising, given the lack of EB shedding in the E2- and E2/P4- exposed mice. Combined with the results from chlamydial titer analysis, the increased T cell presence in the HF- and P4- treated mice suggest that T cells are being recruited to the genital tract in these hormonal conditions in response to *C. muridarum* infection. Interestingly, sham mice, which were treated with Depo-Provera, also had significantly fewer numbers of T cells on day 10pi compared to the HF and P4 groups. These data suggest that Depo-Provera, a synthetic progesterone may illicit different effects on chlamydial pathogenesis and may blunt the immune response to *C. muridarum* compared to natural progesterone.

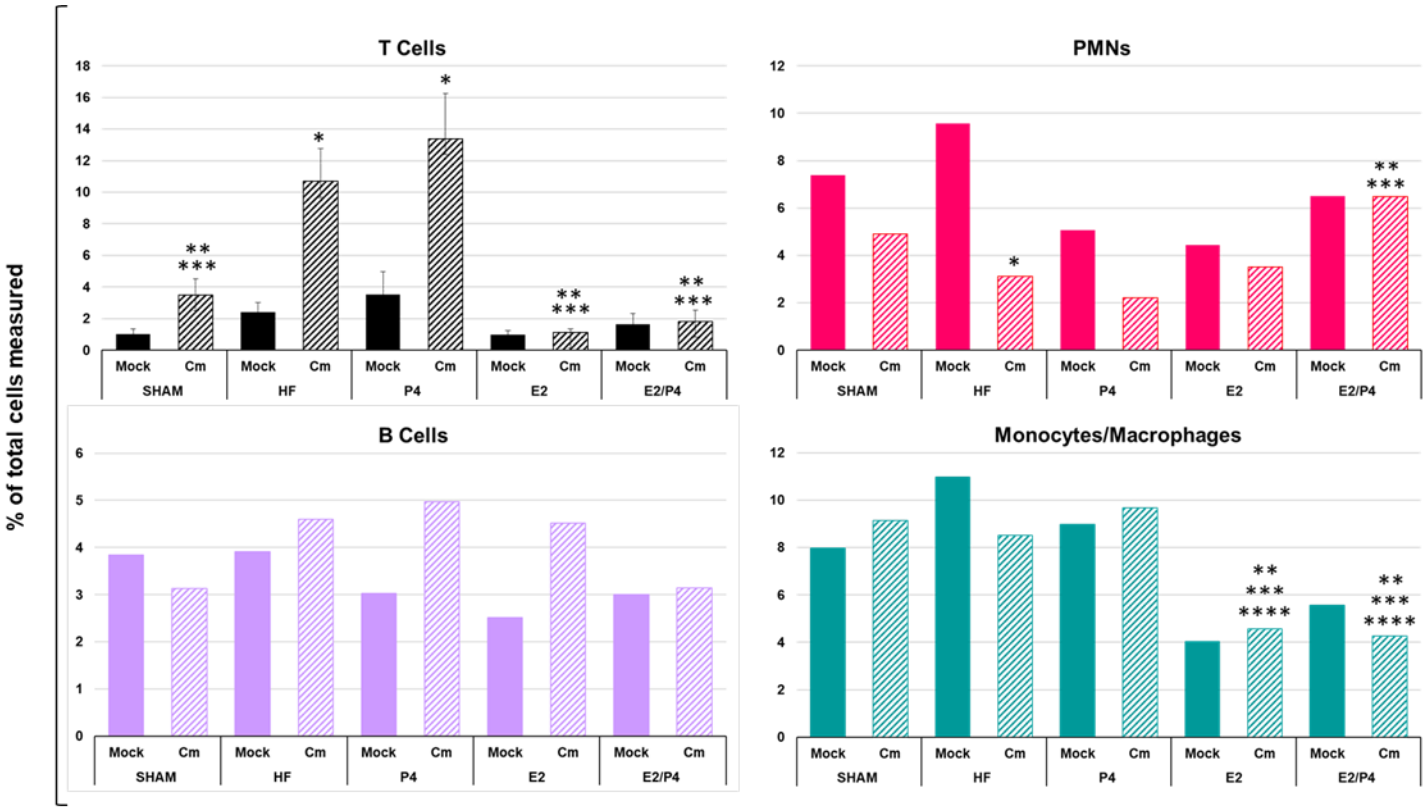

Figure 3.3: Flow cytometry analysis of immune cells. A subset of mock or *C. muridarum* (Cm) mice were sacrificed on day 10pi and flow cytometry was used to measure T cells, B cells, PMNs, and monocytes/macrophages in genital tract tissues. Results are presented as the percentage of total cells measured per group. * Cm vs mock, ** HF(Cm) vs group, ***P4(Cm) vs group, **** sham (Cm) vs group

The absence of ERα in mice alters C. muridarum vaginal shedding

Previous studies have shown that estrogen receptors are expressed differently in various cell types and tissues (Eyster 2016) and that signaling via ERs alter immune cell function and chlamydial infection (Hall et al. 2011). Thus, we wanted to examine the specific effects of ERα and ERβ on chlamydial infection in mice. ERα and ERβ KO mice, along with their respective C57BL/6 WT strains were vaginally infected with *C. muridarum.* Bacterial shedding was monitored for 21 days. ERαWT and ERβWT mice shed EB in a similar pattern, with a peak in EB shedding on day 6pi that decreased by day 9pi (Figure 3.4A). ERβKO mice had a similar shed curve to the WT mice, with the exception of a larger peak in EB shedding on day 6pi. Conversely, peak EB shedding from infected ERαKO mice occurred on day 3pi with no EB collected by day 9 pi. Interestingly, although the ERαKO shed curve was shifted, there was no significant difference in the total number of IFU collected from each group over the total 21-day study period (Figure 3.4B).

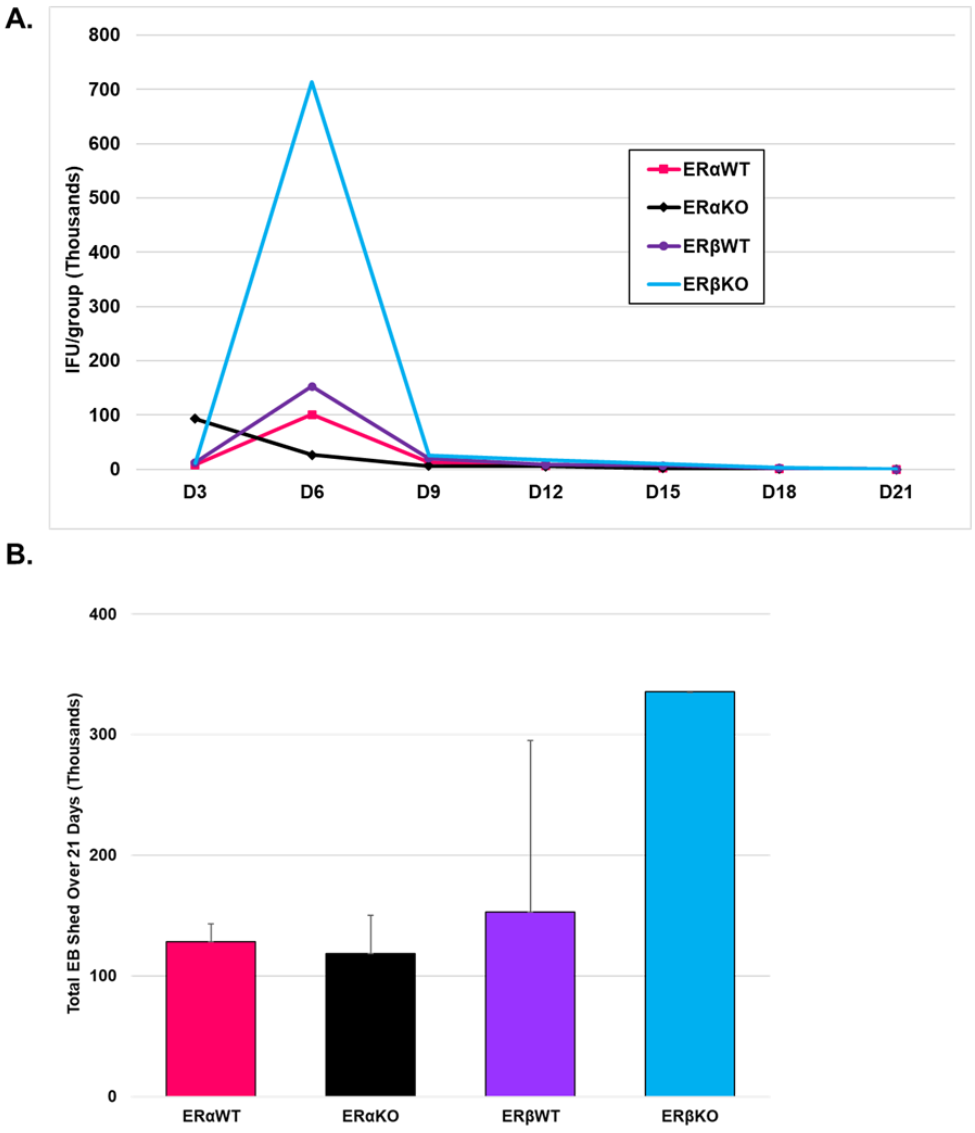

Figure 3.4: Estrogen receptors affect EB shedding. **A.** ERαKO mice had a shed curve that peaked sooner and cleared quicker than the ERβKO and WT groups. While the ErβKO group has a larger peak on day 6pi, this increase in shedding was not significant compared to the other groups. **B.** Total shedding over the 21-day period was compared and there were no significant differences between groups.

ERα and ERβ influence the pathology of the genital tract after chlamydial infection

At the time of sacrifice, genital tracts from all mice were examined *in situ* for gross pathology and assigned a score based on redness and swelling (Table 3.1, Supplemental Figure 3.1). On day 9pi, the genital tracts of infected ERαKO mice were significantly more red and

swollen than the genital tracts of mock-infected ERαKO mice. These high pathology scores from infected ERαKO mice on day 9pi were reduced by day 21pi. The ERαKO mice on day 21pi had significantly less redness and swelling than the WT and ERβKO mice. These data are consistent with EB shedding and correlates with the rapid clearance of infection by ERαKO mice. Pathology scores of the *C. muridarum*-infected ERβKO and WT mice show that their redness and swelling increased from day 9pi to day 21pi, suggesting that the immune response to infection was delayed compared to that of the ERαKO mice (Figure 3.5).

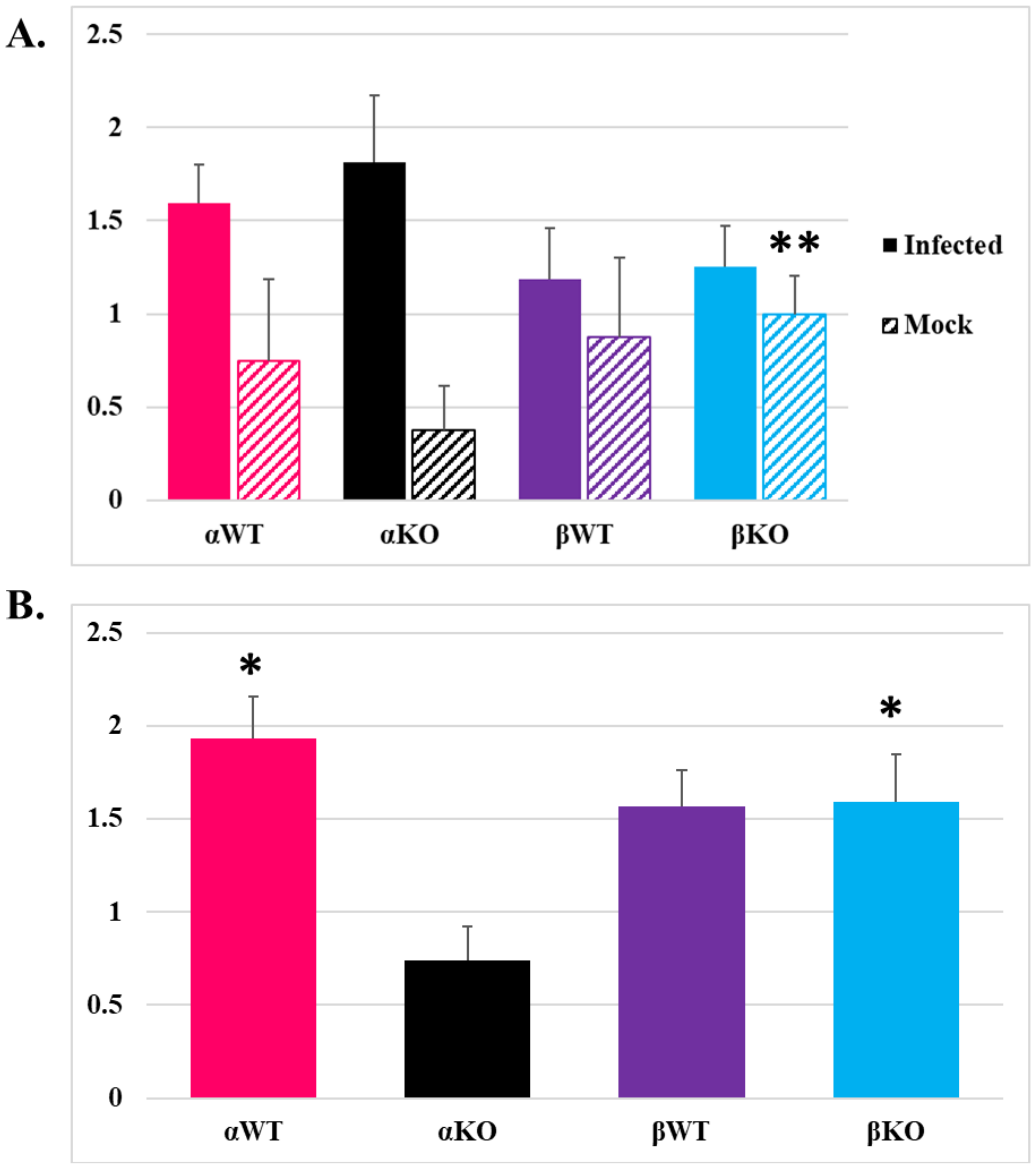

Figure 3.5: Gross pathology of genital tracts at time of sacrifice. Upon sacrifice on day 9 post infection (A) or day 21 post infection (B), genital tracts were observed and given a score based on redness and swelling. * significantly different than ERαKO infected, ** significantly different than ERβKO infected.

The T cell response is altered in ERαKO mice

To examine the immune cell response to *C. muridarum* infection in C57BL/6 mice, we collected genital tracts from mock and infected WT and ERKO mice on day 9pi. We chose this

time as it represented the initial decrease in EB shedding from infected mice, it has been reported that *C. muridarum* ascends the genital tract by this time (Darville and Hiltke 2010; Murthy et al. 2018), and other studies on immune response to *Chlamydia* showed establishment of immune cells by 6-10 days post infection (Frazer et al. 2013; Campbell et al. 2014; Poston et al. 2017). We used flow cytometry to examine the proportion of myeloid and lymphocyte immune cells present in genital tract at that time.

At day 9pi, ERαKO mice infected with *C. muridarum* had significantly more T cells present in the genital tract than infected ERβKO mice and infected WT mice. ERαKO infected mice also had significantly more T cells present than their corresponding mock-infected group (figure 3.6). These data indicate that ERα is involved in the regulation of the T cell response to chlamydial infection in mice.

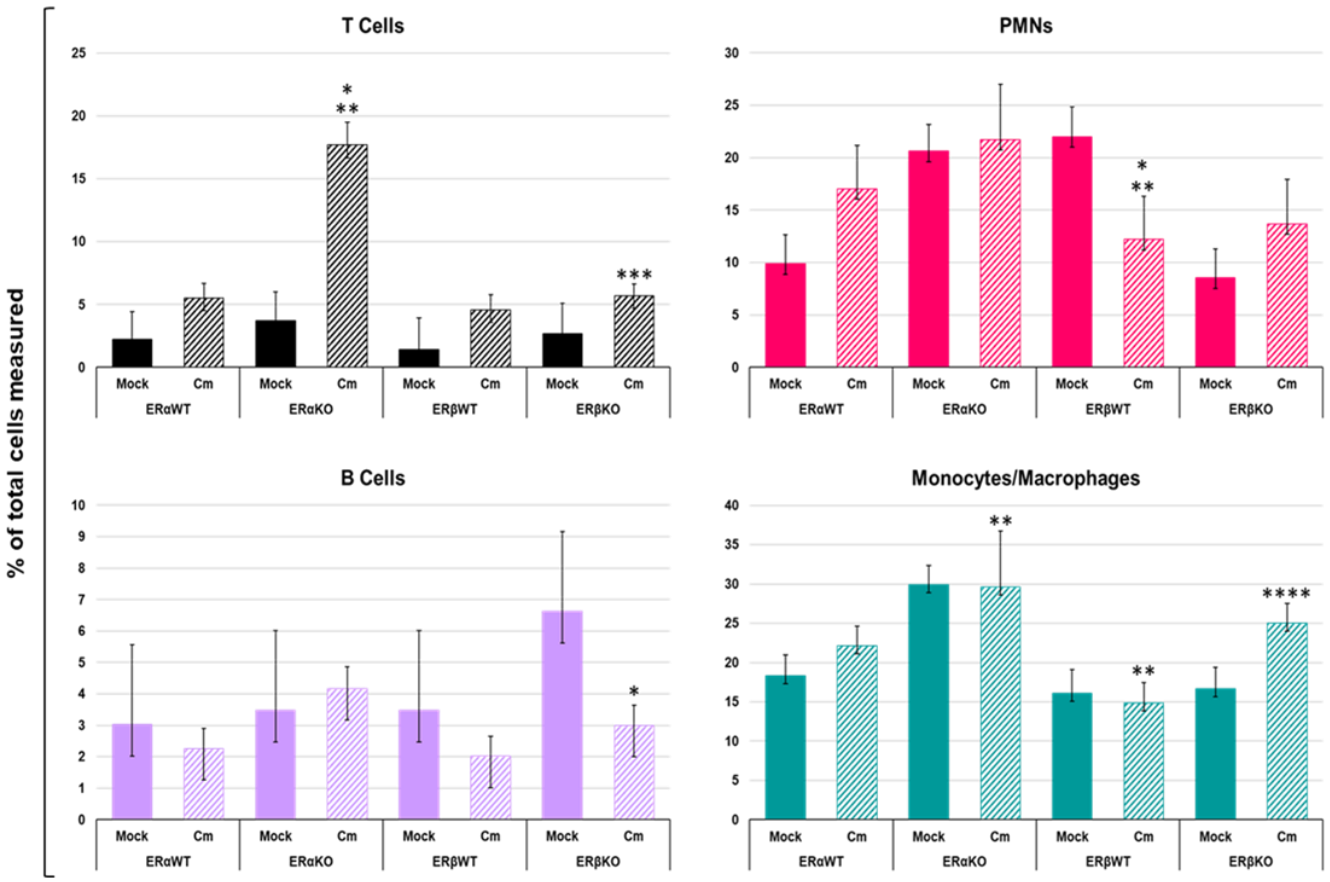

Figure 3.6: Flow cytometry analysis of immune cells. Mock or *C. muridarum* (Cm) infected mice were sacrificed on day 9pi and flow cytometry was used to measure T cells, B cells, PMNs, and monocytes/macrophages in genital tract tissues. Results are presented as the percentage of the total cells measured per group. *significant difference vs group's mock, **significant difference vs ERαWT Cm, *** significant difference vs ERβWT Cm, ****significant difference vs ERβWT mock

To further investigate the immune response to *C. muridarum* infection in mice, we examined the expression of 19 immune marker genes in mock and infected WT and ERKO tissues collected at day 9pi using Illumina TruSeq analysis (Table 3.2). Gene expression was compared in full genital tract (GT) tissues as well as in separated tissues for the cervix, horn, and ovary. Each group was analyzed, and comparisons were made between tissues as well as between mouse strains.

Table 3.2: Genes Analyzed by RNASeq	
Gene Name	Abbreviation
BCL6 Transcription Repressor	BCL6
Cluster of Differentiation 4	CD4
Cluster of Differentiation 8a	CD8a
C-X-C motif ligand 15	CXCL15
EGF-like module-containing mucin-like hormone receptor-like 1	EMR1 (aka F4/80)
Estrogen receptor alpha	ESR1
Estrogen receptor beta	ESR2
Forkhead box P3	FOXP3
Interferon alpha-1	IFNA1
Interferon beta	IFNB1
Interferon gamma	IFNG
Interleukin-17a	IL17A
Interleukin 2	IL2
Interleukin 4	IL4
Eosinophil major basic protein	PRG2
Transforming growth factor beta 1	TGFB1
Toll-like receptor 2	TLR2
Toll-like receptor 4	TLR4
Tumor necrosis factor	TNF

As expected, the expression of inflammatory cytokines IFNB1, IFNG, and TNF were increased in infected WT mice compared to mock-infected WT mice. The majority of the significant changes observed in immune-related gene expression were between the ERαKO and WT groups (Figure 3.7A). When comparing ERαKO to WT GT, we saw that the ERαKO mice had upregulated CD4 and FOXP3 expression, indicating that the absence of ERα resulted in increased helper T cells, specifically regulatory T cells (Tregs). Conversely, we observed downregulated levels of CXCL15 and ESR1 expression in ERαKO verses WT GT. ESR1 encodes ERα, thus the downregulation of this gene was expected in ERαKO tissues. ESR1 was also significantly decreased in both the horn and ovary of ERαKO verses WT mice. In addition to the full GT, expression of CXCL15, which encodes IL8 in mice, was decreased in the cervix and ovary of ERαKO mice compared to WT. IL2 expression was differentially altered, being increased in the ovary and decreased in the cervix of ERαKO compared to WT. Cytokine expression in the horn of ERαKO mice was decreased with significantly less IFNB1 expression than WT mice. Lastly, the ovary of ERαKO mice had significantly more helper T cell (CD4) and eosinophil (PRG2) expression than the WT mice.

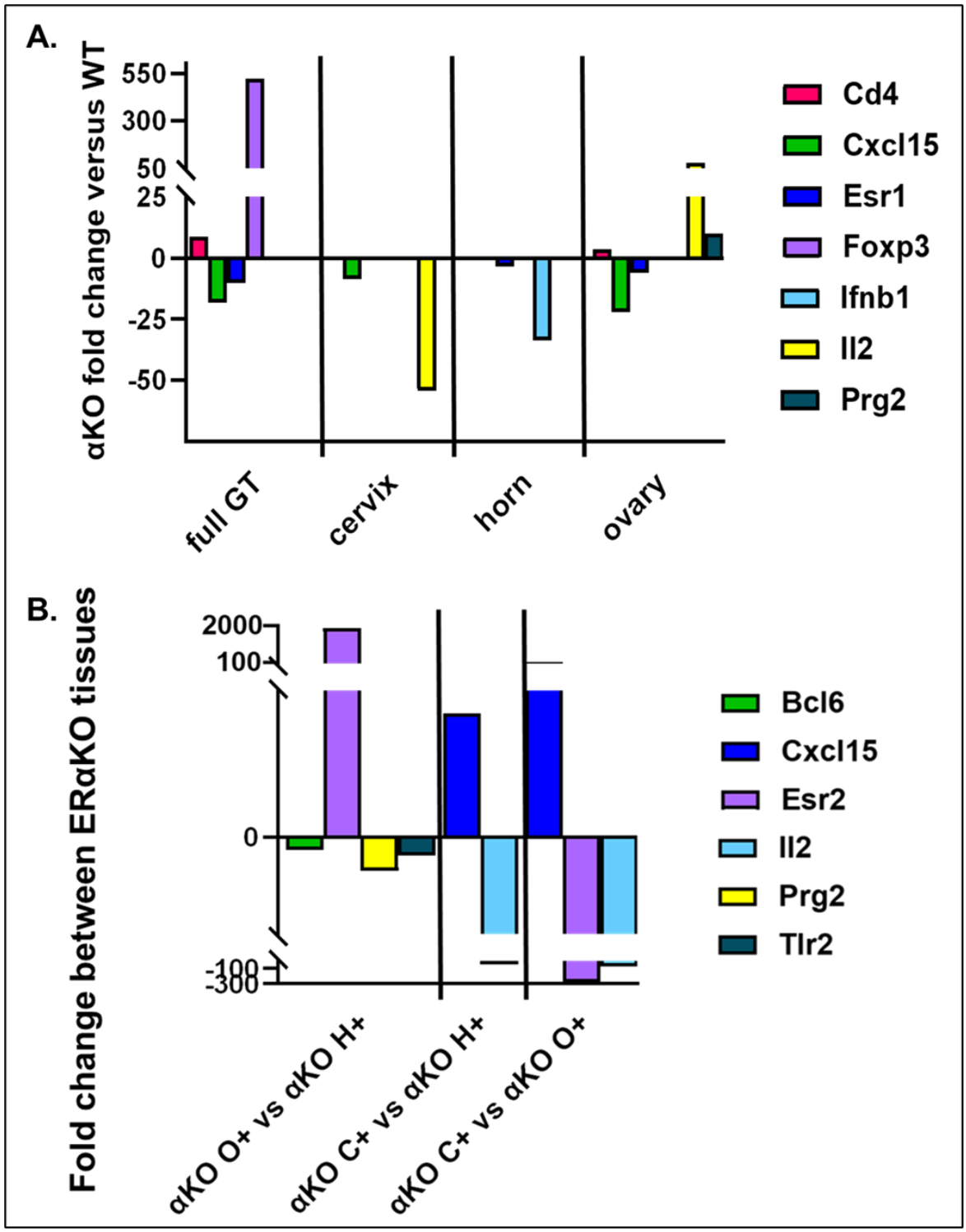

Figure 3.7: Fold change values of genes between ERαKO and WT mice. Results show the fold change value of genes determined to be significantly different by differential expression of RNASeq tool in the CLCBio Genetics Workbench. **A.** Fold changes between ERαKO and WT genital tract (GT), cervix, horn, and ovary **B.** Fold changes between ERαKO tissues, ovary (O), horn (H), and cervix (C).

We also observed changes between the separate ERαKO tissues (cervix, horn, and ovary) (Figure 3.7B). In infected ERαKO mice, we saw that the cervix had upregulation of CXCL15 and downregulation of Il2 compared to the horn and ovary, and downregulation of ESR2 compared to the ovary. Additionally, the ovary had increased ESR2 and decreased BCL6, PRG2, and TLR2 compared to the horn

Discussion

Previous studies from our lab demonstrated that 17-β estradiol (E2) treatment enhanced *C. trachomatis* infection in an IK/SHT290 co-culture model, and that progesterone (P4) antagonized the enhancement observed withE2-only exposure (Kintner et al. 2015). Further, E2 was found to increase attachment of *C. trachomatis* EB to human epithelial cells, while P4 reduced attachment (Maslow et al. 1988). Based on these data, we sought to examine the effects of E2 and P4 on chlamydial infection *in vivo.*

There are several reports that E2 and P4 treatment affect rats and mice differently than humans, swine, and guinea pigs. Treatment of animals with E2 has various effects on chlamydial infection. *C. caviae* is a species of *Chlamydia* that causes inclusion conjunctivitis in guinea pigs, known as guinea pig inclusion conjunctivitis (GPIC). E2 treatment of guinea pigs prior to *C. caviae* infection resulted in a longer lasting infection with higher numbers of EB collected from vaginal swabs, as well as more pathology than non-E2 treated guinea pigs (Rank et al. 1982). Further, genital epithelial cells isolated from swine exhibited greater chlamydial attachment and infectivity when the cells were collected during the estrogen-dominant phase of the animal's estrus cycle (N V Guseva et al. 2003). Conversely, the treatment of rats and mice with E2 prior to chlamydial infection results in the complete protection from infection, and these animals

require P4 treatment for successful infection of the genital tract (Kaushic et al. 1998; Kaushic et al. 2000). However, because they are not ovariectomized, E2-treated mice in previous studies continue their estrus cycle, preventing chlamydial infection. Additionally, these studies did not measure serum hormone levels in animals after E2 treatment to ensure that physiological levels were achieved. In contrast, we used OVT mice that would no longer produce endogenous E2 and P4 and used LC/MS-MS to confirm that our mice had physiologically relevant levels of circulating E2 and/or P4.

The short estrus cycle in mice prevents genital *Chlamydia* infection from being established in actively cycling mice. Progesterone in the form of depomedroxyprogesterone acetate (DMPA, Depo-Provera) is widely used in the field of chlamydial research as a pretreatment to stop the estrus cycle before chlamydial infection by causing mice to be suspended in the diestrus phase (Kaushic et al. 2003). Therefore, existing literature highlights the necessity of progesterone treatment in the form of DMPA to establish chlamydial infection. This model, however, does not allow for investigation of the effects that naturally occurring progesterone- or estrogen-dominant environments have on chlamydial infection in mice. DMPA treatment of mice has previously been shown to reduce antibody response to HSV-2 following immunization compared to P4 treatment (Kaushic et al. 2003). Our current study demonstrates that DMPA-treated mice had reduced T cells present on day 10pi compared to HF or P4-treated OVT mice. These findings suggest that DMPA influences the immune response to infection differently than endogenous progesterone. DMPA may actually suppress the immune response to infection in the female genital tract, highlighting the need for a model of chlamydial infection that does not require pretreatment with DMPA.

The current study aimed to explore the interactions between female sex hormones, estrogen receptors, *C. muridarum* and host immunity using two murine models of chlamydial infection, estrogen receptor knockout mouse strains and hormone supplemented OVT mice. Using the silastic capsule method of hormone administration, we were able to introduce physiologically relevant levels of E2 and P4 to OVT mice in order to study hormone-specific features of chlamydial pathogenesis and host immunity. We saw that physiologically relevant levels of E2, even in the presence of P4, completely protected mice from *C. muridarum* infection. A previous study infecting E2-, P4-, or combination E2/P4-treated OVT rats with *C. muridarum* reported that E2 completely protected the rats from chlamydial shedding and inflammation while P4 treatment resulted in greater than 10^9 IFU/ml detected in vaginal washes and severe inflammation in the genital tracts. However, this study found that combination E2/P4 treatment of these rats prior to chlamydial infection did not protect the rats from infection, measured by chlamydial shedding, although the combination-treated rats did have reduced inflammation compared to the P4-treated rats (Kaushic et al. 2000).

Our experiments with ERα and ERβ knockout mice showed that the absence of ERα, but not ERβ, resulted in an earlier peak in EB shedding compared to the WT groups. While the ERβKO and WT groups peaked in EB shedding on day 6pi, peak shedding from ERαKO mice occurred on day 3pi. This suggests that the lack of ERα signaling resulted in a quicker progression of chlamydial infection. The mechanism behind this change in chlamydial pathogenesis in the absence of ERα is not clear. Despite being similar in structure and in function, ERα and ERβ are located on different chromosomes and are expressed differentially on various tissues throughout the body. In mice and in humans, ERα is highly expressed in the ovaries as well as the uterus. Conversely, ERβ is the dominant ER in the ovaries, but is not

expressed in the uterus (Wang et al. 2000; Jia et al. 2015; Hamilton et al. 2017). Analysis of gene expression in *C. muridarum* infected ERαKO mice indicted that ERβ was not upregulated in the horn to compensate for the loss of ERα. Additionally, ERs are expressed differentially on immune cells. In humans, ERα and ERβ are expressed on most immune cells at varying concentrations (Kovats 2015). ERα has been reported as having higher expression on CD4+ T cells compared to ERβ, while ERβ was more highly expressed on B cells. The same study reported no differences in ER expression in Th1 and Th2 cells (Phiel et al. 2005). In mice, ERα but not ERβ was detected in macrophages, CD4+ and CD8+ T cells (Lambert et al. 2005).

ERs can both enhance and dampen innate immune cell function. Studies have shown that E2 increases FOXP3 expression *in vitro* and *in vivo* (Tai et al. 2008; Moore-Connors et al. 2013) and decreases CXCL15 expression (Mircheff et al. 2010 Jan 1). E2 signaling also increases IFNγ production (Nakaya et al. 2006) but dampens IL-2 production (McMurray et al. 2001). These examples provide a small insight to the complexity of E2 signaling on immune function, and further studies are required to gain understanding of how these interactions influence chlamydial infection.

Our data suggests that ERα is involved in the resolution of chlamydial infection through its interactions with the immune system. We found that in the absence of ERα, the timing of chlamydial shedding progressed faster than in WT mice or in the absence of ERβ. These data correlate with the pathology scores, which showed that ERαKO mice had high amounts of swelling on day 9pi which was resolved by day 21pi. However, the ERβKO mice and the WT mice had high pathology scores on day 21pi, indicating that while chlamydial shedding had resolved, these mice still had inflamed genital tracts at the end of our study period. Additionally, there were no significant differences observed in the myeloid population of immune cells, but the

lymphocyte population in the ERαKO mice was significantly altered compared to the ERβKO and WT mice. *C. muridarum*-infected ERαKO mice had significantly higher numbers of T cells present in the genital tract compared to their corresponding mock-infected mice, ERβKO mice, and WT mice. E2 treatment has been shown to increase FOXP3 expression in WT mice compared to ERαKO mice, suggesting that ERα signaling was responsible for the E2-mediated FOXP3 increase (Polanczyk et al. 2004). This disagrees with our finding that ERαKO mice had a significant increase in T cells and FOXP3 expression during *C.muridarum* infection.. One may expect that increased Treg presence could inhibit immune action against infection due to their regulatory nature. However, previous studies showed that Treg production was actually increased in the presence of *C. muridarum* infection *in vitro* and in mice, and possibly served to encourage the production of Th17 to aid in the clearance of chlamydial infection (44). Given these observations our study suggests that ER signaling, primarily ERα, alters the progression of *in vivo C. muridarum* infection via regulation of the T cell response.

Conclusions And Future Direction

Together, our data indicate that E2 and ERs have important roles in the pathogenesis of *C. muridarum* infection in mice. One limitation of this study is that our observation that the immune response to chlamydial infection is regulated by hormones and ER signaling was limited to one time during *C. muridarum* infection. Another possibility is that ER signaling in infected epithelial cells contributes to the observed alterations in chlamydial infection as has been demonstrated *in vitro* (Kovats 2015). Examining the immune response at multiple time points throughout the infection period would provide more information about why EB shedding in ERαKO mice peaked and resolved faster than that observed in ERβKO and WT mice.

Additionally, our studies examined the role of individual ER on chlamydial infection. Future studies, investigating chlamydial infection in a double ERα/ERβ knockout murine model or by treating ERαKO mice with an ER antagonist would provide a better understanding of the roles ERs play in regulating chlamydial pathogenesis. Overall, these data reiterate the importance of estrogen-stimulated signaling on chlamydial pathogenesis and highlight the need for future investigations to deepen our understanding about the ways sex hormones influence STI pathogens.

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CHAPTER 4. LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY APPLICATIONS FOR QUANTIFICATION OF ENDOGENOUS SEX HORMONES

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Keywords: Steroid hormone bioanalysis; estradiol; progesterone; testosterone; LC-MS/MS

Abstract

Liquid chromatography, coupled with tandem mass spectrometry, presents a powerful tool for the quantification of the sex steroid hormones 17-β estradiol, progesterone and testosterone from biological matrices. The importance of accurate quantification with these hormones, even at endogenous levels, has evolved with our understanding of the role these regulators play in human development, fertility and disease risk and manifestation. Routine monitoring of these analytes can be accomplished by immunoassay techniques, which face limitations on specificity and sensitivity, or using gas chromatography–mass spectrometry. LC–MS/MS is growing in capability and acceptance for clinically relevant quantification of sex steroid hormones in biological matrices and is able to overcome many of the limitations of immunoassays. Analyte specificity has improved through the use of novel derivatizing agents, and sensitivity has been refined through the use of high-resolution chromatography and mass spectrometric technology. This review highlights these innovations, among others, in LC–MS/MS steroid hormone analysis captured in the literature over the last decade.

1. Introduction to Sex Steroid Hormone LC–MS

Endogenous sex hormones, such as testosterone and estrogen, play vital roles in the coordination and normal development of both male and female sex characteristics. These hormones are important chemical messengers derived from cholesterol and undergo synthesis following complex metabolic pathways, as shown in Figure 4.1 (Galligan et al., 2018). The hormonal milieu of endogenous sex hormones has been thought to play a role in multiple disease processes, such as infertility, osteoporosis, cardiovascular disease, breast cancer, prostate cancer and endometrial cancer (Hankinson & Tworoger, 2011; Naessen et al., 2010). Recently, testosterone levels have been linked to increased SARS-CoV-2 transmission in males; therefore, examining these complex hormones has become important in epidemiological and metabolomic investigations (Montopoli et al., 2020). Their correct identification is paramount to clinical practice and patient care.

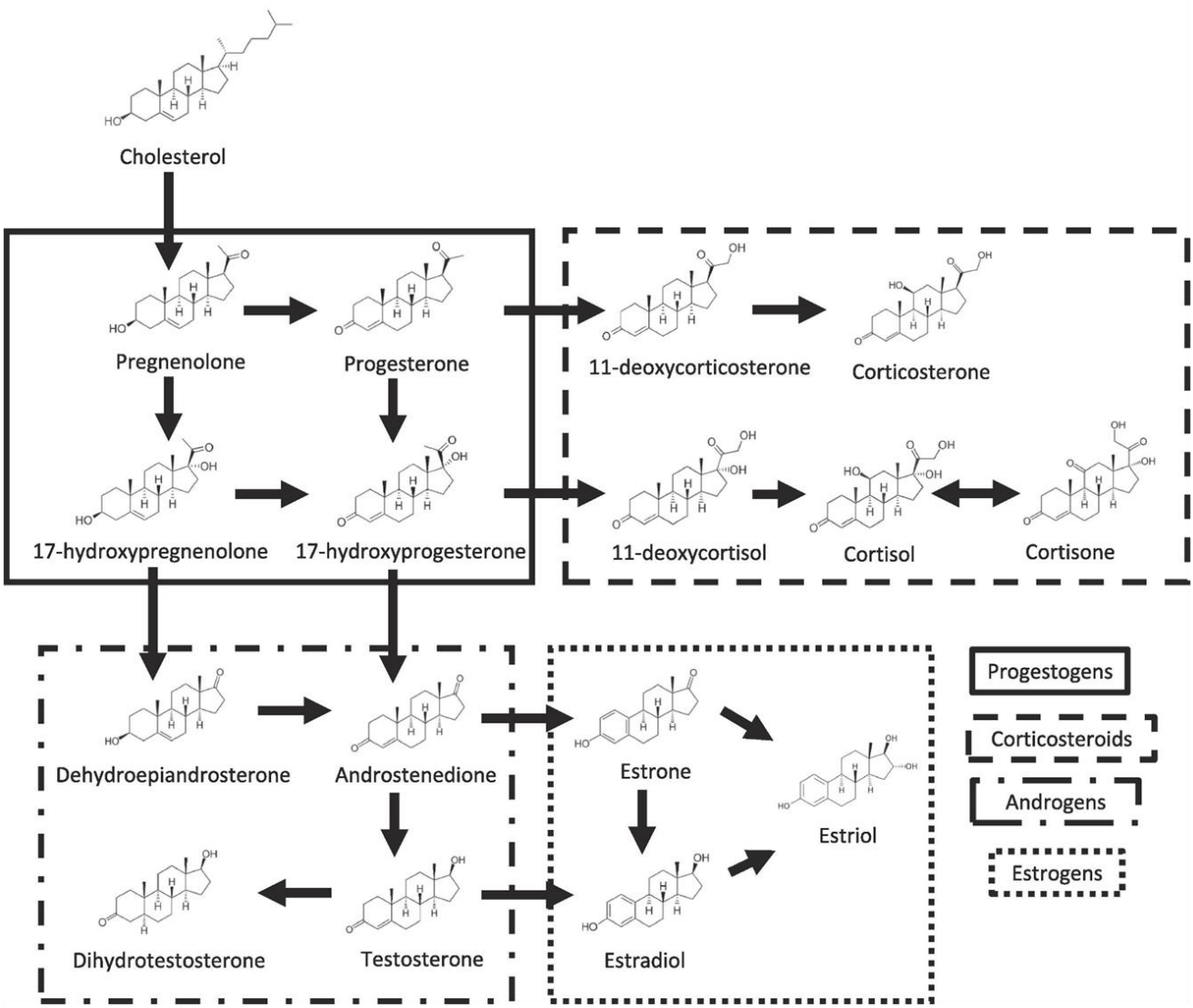

Figure 4.1: Steroidogenesis pathway. Reprinted (figure 1) with permission from Galligan, T. M., Schwacke, L. H., Houser, D. S., Wells, R. S., Rowles, T., & Boggs, A. S. P. (2018). Characterization of circulating steroid hormone profiles in the bottlenose dolphin (Tursiops truncatus) by liquid chromatography–tandem mass spectrometry (LC–MS/MS). General and Comparative Endocrinology, 263, 80–91. https://doi.org/10.1016/j.ygcen. 2018.04.003

Prior to advancements in high performance liquid chromatography–mass spectrometry (HPLC–MS), the predominant methods to measure endogenous sex hormones were conventional

and/or direct radioimmunoassay (RIAs). However, these methods have many limitations. For example, conventional RIAs are costly, time-consuming, require large sample volumes and are subject to antibody cross-reactivity (Stanczyk, Jurow, & Hsing, 2010). Conversely, direct RIAs often overestimate measurements owing to lack of specificity of antibodies and lack of reliability/sensitivity to detect low-level steroid hormones such as 17-β estradiol, and can only measure one analyte at a time (Tate & Ward, 2004). The previously described limitations of RIAs have restricted their utility largely to analytes of single steroids or steroid metabolites present in high volumes, rather than steroid profiles or samples of closely related steroids (Abraham, 1975).

Advancements in MS development have followed an exciting timeline which has led to the widely used current practice of LC–MS for the measurement of hormones (Shackleton, 2010). Mass spectrometry has transitioned over the past 20 years toward the status of "gold standard" for hormone analysis (Conklin & Knezevic, 2020; Wudy & Choi, 2016). Setting the stage for the practical use of MS studies in hormone measurement was the gas-chromatographic (GC) separation of sterols in the 1960s (Eneroth, Hellstroem, & Ryhage, 1964). Gas chromatography–mass spectrometry (GC/MS) evolved as a powerful tool for the identification and quantification of unconjugated steroids. It remains indispensable in defining steroid disorder metabolomes but is limited by the need for derivatization to promote volatility, which negatively impacts sample throughput (Bloem et al., 2015; Krone et al., 2010).

With a goal of identifying polar compounds without the need for chemical derivation, the 1980s saw the introduction of fast atom bombardment (FAB). This was the first MS technique to make this analysis possible (Barber, Bordoli, Sedgwick, & Tyler, 1981). The technique utilizes ambient temperature and involves a nonvolatile matrix as a sample target, which following

placement in the ion source, is bombarded by a stream of atoms, e.g. Xe. This is subsequently ionized and detected using a mass spectrometer (Barber et al., 1981). Fast atom bombardment technology also progressed in the development of the use of continuous flow, allowing online HPLC FAB-MS and tandem MS instrumentation to further identify and quantify conjugated sterols (Barber et al., 1981). However, this groundbreaking technique was eventually surpassed by the introduction of HPLC–MS.

HPLC–MS was first described utilizing thermospray as the ion source in the early 1980s (Blakley & Vestal, 1983). The technique became suitable for the analysis of both conjugated and unconjugated sterols, but the ion source would ultimately be superseded by the development of electrospray ionization (ESI) mass spectrometry (Yamashita & Fenn, 1984). Atmospheric pressure chemical ionization (APCI) evolved as a complimentary technique to electrospray ionization (ESI) and is relevant in steroid mass spectrometry owing to its reduced potential for matrix effects vs ESI (Kushnir et al., 2011). More recently, atmospheric pressure photoionization (APPI), which uses a dopant solvent to facilitate proton transfer to nonpolar analytes, has also become an important ion source in steroid mass spectrometry assays also seeking to minimize matrix effects (Hanold, Fischer, Cormia, Miller, & Syage, 2004; Robb, Covey, & Bruins, 2000). Since its inception, HPLC coupled with tandem mass spectrometry (LC–MS/ MS) has revolutionized the quantification of steroid hormones and quickly became the technique of choice in both clinical and research laboratories. This technique allows for a wider range of analytes than GC–MS and has recently been associated with the discovery of novel metabolites in the steroidogenesis pathway (Bloem et al., 2015). The further development of reference standardization in the field of hormone quantification, coupled with the specificity, sensitivity and multiplexing capabilities inherent to LC–MS/MS, will employ this technique to remain a

dominant methodology for hormone quantification in the clinical and research setting (Seger & Salzmann, 2020).

The purpose of this review was to summarize some of the trends in sex steroid hormone quantification over the past 10 years, including testosterone, progesterone and 17-β estradiol. The structures of these hormones can be seen in Figure 4.2 (Conklin & Knezevic, 2020). Despite a history of mass spectrometric analysis that begins in the 1960s, steroid bioanalysis is and remains a challenging undertaking. Structural similarities among the analytes, as demonstrated in Figure 1 (Galligan et al., 2018), coupled with limited ionizability, limited unique fragmentation and low numbers of heteroatoms represent some of the many challenges. To gather articles included in this review, the authors utilized Science Direct, Wiley Online Library, PubMed and Google Scholar to identify articles that presented research related to each hormone and LC–MS. Search terms included "mass spectrometry + hormone name", "LC–MS + hormone name" and "chromatography + hormone name" for each analyte of interest. Most of the results included in this review date back to 2010, but some references are older to provide appropriate historical context, or where the method was particularly impactful. The articles were further culled to exclude those without biological matrices.

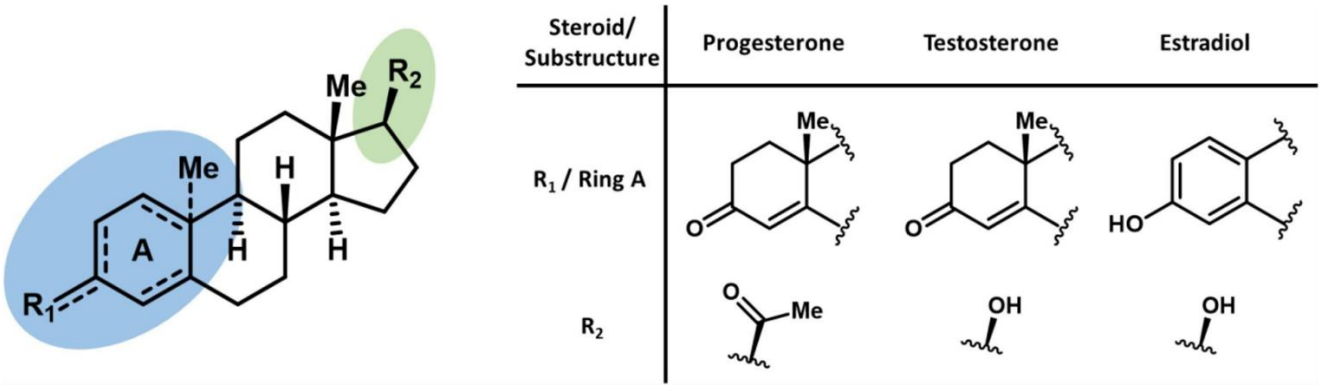

Figure 4.2: Structures of progesterone, testosterone, and estradiol. reprinted (Figure 1) with permission from Conklin, S. E., & Knezevic, C. E. (2020). Advancements in the gold standard: Measuring steroid sex hormones by mass spectrometry. Clinical Biochemistry, 82, 21–32. https://doi.org/10.1016/j. clinbiochem.2020.03.008

2. *Testosterone Bioanalysis*

Quantification of testosterone in biological samples has many clinical and research applications, including hormone profiling in animals (Farke, Rattenberger, Roiger, & Meyer, 2011; Hauser, Deschner, & Boesch, 2008; Holst, Kushnir, & Bergquist, 2015; Kaabia, Laparre, Cesbron, Le Bizec, & Dervilly-Pinel, 2018; Kaufmann, Butcher, Maden, Walker, & Widmer, 2019; Legacki, Robeck, Steinman, & Conley, 2020; Shackleton, 2010), hormone profiling in humans (Häkkinen et al., 2018; Zheng, Zhao, Zhu, & Cai, 2019) and disease investigation (Kushnir et al., 2009; Lood et al., 2018; Yuan et al., 2019). Being able to accurately measure testosterone concentrations in human samples is essential in accurate diagnoses of hypogonadism, polycystic ovary syndrome, and certain cancers (Bhasin et al., 2010; Ka, 2016; Loblaw et al., 2007; Wierman et al., 2006). Clinically relevant testosterone assays apply to ranges of 20–70 ng/dL (females) and 300–1,000 ng/ dL in males. Additionally, most testosterone in circulation is protein bound (Mezzullo et al., 2020; Tavita & Greaves, 2017).

Historically, this quantification was accomplished by immunoassay or gas chromatography–mass spectrometry (GC–MS) (Rosner, Auchus, Azziz, Sluss, & Raff, 2007), but as the needed limits of sensitivity reach the endogenous range, the immunoassay approach common in the clinical laboratory has been criticized as equally accurate to "guessing" (Herold & Fitzgerald, 2003). Several investigators have demonstrated the limitations of immunoassays for testosterone quantification by comparing their results with those obtained using GC– MS or LC–MS (Chen, Yazdanpanah, Hoffman, Diamandis, & Wong, 2009; Hogg, Vickers, & Rogers, 2005; Moal, Mathieu, Reynier, Malthièry, & Gallois, 2007; Owen, Rawlins, & Roberts, 2010; Taieb et al., 2003; Wang, Catlin, Demers, Starcevic, & Swerdloff, 2004; Wooding et al., 2015). These limitations prompted an official statement from the Endocrine Society promoting a mass spectrometry-based approach as the "gold standard" for testosterone measurements (Rosner et al., 2007).

To address standardization for assays for testosterone and other hormones, the Centers for Disease Control (CDC) initiated the Hormone Standardization Program (HoSt) in 2007 (French, Drees, Stone, Holmes, & van der Gugten, 2019). The CDC used the National Institute of Standards and Technology (NIST) Reference Measurement Procedure (RMP) for testosterone as a benchmark to develop their own standardization (Botelho et al., 2013; Tai, Xu, Welch, & Phinney, 2007; Vesper et al., 2014). In addition to CDC's HoSt program, NIST also offers a Standard Reference Material (SRM 971) to assist external laboratories with accurate testosterone quantification (French et al., 2019).

The NIST assay for testosterone is based on a serum matrix, where a combination of solid-phase extraction (SPE) and liquid–liquid extraction (LLE) with hexane is used to prepare the sample (Tai et al., 2007). A deuterium labeled testosterone (d3) internal standard was used to

facilitate quantification by LC–MS/MS. Separation was achieved under reversed-phase conditions (Zorbax Eclipse XDB-C_{18}). During the method development, this assay was applied to quantifying testosterone from male and female donors, achieving a limit of detection of 2 pg (Tai et al., 2007). Of note, testosterone quantification in females can be especially problematic for existing immunoassays. One comparison of immunoassay with mass spectrometry found that the immunoassay overestimated the testosterone concentration 5-fold (Taieb et al., 2003). Despite the long run time (40 min), the assay developed and validated by NIST demonstrates noninterference from 21 structural analogs (Tai et al., 2007). The CDC method, developed as part of HoSt, also utilizes a serum matrix, prepared with a combination of protein precipitation (ammonium acetate) and LLE (ethyl acetate–hexane) (Botelho et al., 2013). Reversed-phase separation (C_{18} Hypersil Gold) was used in conjunction with +ESI-LC–MS/ MS. In contrast to NIST, the CDC approach utilizes isotope dilution with [$^{13}C_3$]-testosterone (Botelho et al., 2013; Tai et al., 2007). Despite having the same parent mass and sometimes the same productions, 18 structural analogs were chromatographically separated from testosterone, thus producing no interference with the selected reaction monitoring (SRM) transitions (m/z 289→97 for quantification and m/z 289→109 for confirmation) (Botelho et al., 2013).

3. Estradiol Bioanalysis

17-β-Estradiol is the predominant circulating female sex steroid hormone. While it circulates in large amounts in premenopausal women, it also circulates in small amounts in post-menopausal women, men and children. Regarding quantification of 17-β-estradiol, the Endocrine Society released a statement in 2013 stating “the measurement of estradiol in biological fluids is important in human biology from cradle to grave” (Rosner, Hankinson, Sluss, Vesper, & Wierman, 2013). This hormone is part of the CDC's HoST program, yet the goals of assay

standardization for 17-β-estradiol have not been as successfully met as they have for testosterone (Seger & Salzmann, 2020; Vesper et al., 2014). For the last 30-plus years, immunoassays have been used for the measurement of 17-β-estradiol; however, in recent decades it has become increasingly clear that immunoassays are not sensitive or accurate enough to be relied upon by clinicians. Furthermore, clinician awareness regarding assay limitation, especially at 17-β-estradiol concentrations <50 pg/ml, may limit the applicability of laboratory data in clinical practice (Demers, 2008). Owing to issues with cross-reactivity, immunoassays have the potential to overestimate estradiol concentrations by up to 60% (Gao, Stalder, & Kirschbaum, 2015). One historical source of cross-reactivity for estradiol immunoassay accuracy is the presence of contraceptive steroids in the sample (Kharma, Stone, Thorneycroft, Nakamura, & Mishell, 1972).

Accurate quantification of 17-β-estradiol has several clinical applications. Many laboratories have the capability to measure estradiol at the levels needed to support women seeking fertility assistance, but these levels tend to exceed 50 pg/ml (Demers, 2008). Large dynamic ranges, up to 3,000 pg/ml, may be necessary to accommodate this population (Rosner et al., 2013). The low-end sensitivity is more applicable to post-menopausal women with median concentrations around 15 pg/ml (Demers, 2008; Stanczyk et al., 2010). Furthermore, management of patients with breast cancer using aromatase inhibitors to suppress 17-β-estradiol levels requires differentiation between 17-β- estradiol levels of <1 pg/ml from pretreatment levels (10–15 pg/ml) (Rosner et al., 2013). Quantification of 17-β-estradiol is further complicated by a need for specificity related to the conversion of this hormone to over 100 conjugated and unconjugated metabolites (Lee, Cai, Thomas, Conney, & Zhu, 2003; Lépine et al., 2004). In the development of an RMP for the determination of 17-β-estradiol from human

serum, NIST utilized extensive sample preparation including two types of SPE (reversed-phase and anion exchange) followed by derivatization with dansyl chloride (Tai & Welch, 2005). Similar to their approach for testosterone, the application of isotope dilution coupled with LC–MS/MS helped achieve validation of an assay that meets requirement of an RMP, with cross-checked validity to the "gold standard" GC–MS/MS assay using Certified Reference Materials (CRM) 576, 577, and 578 (Tai et al., 2007; Tai & Welch, 2005; Thienpont, DeLeenheer, & Dirscherl, 1997). Despite this, the detection limit for the NIST method translates to 1 ng/L (Tai & Welch, 2005). Nevertheless, investigators in clinical endocrinology describe high-sensitivity LC–MS/MS as the "path ahead" for estradiol quantification in biological samples (Ketha, Girtman, & Singh, 2015).

4. Progesterone Bioanalysis

Progesterone is a sex hormone derived from cholesterol and is an essential precursor for glucocorticoids and mineralocorticoids. Progesterone levels are often actively monitored in patients seeking fertility assistance, as its role in ovulation is well characterized (Ciampaglia & Cognigni, 2015; Quaas & Hansen, 2016). Furthermore, this hormone plays a role in pregnancy maintenance, and exogenous progesterone may be administered during early pregnancy to counteract deficiencies (Coomarasamy et al., 2015; Quaas & Hansen, 2016; Ransom & Murtha, 2012). Additionally, evidence to support the role of progesterone in cognition and its neuroprotective effect in traumatic brain injury in animals has recently surfaced (Henderson, 2018; Stein & Wright, 2010). Finally, abnormal progesterone levels have been implicated in increased risk of some autoimmune diseases in females, including systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, autoimmune thyroid disease and some other little-known autoimmune diseases such as Sjögren syndrome (Hughes, 2012). Normal levels of progesterone

in women range from 0.15 to 25 ng/ml but may be as high as 230 ng/ml in pregnancy (Mezzullo et al., 2020; Tai, Xu, & Welch, 2006; Tavita & Greaves, 2017). Progesterone levels in women are highest during the middle of the menstrual cycle (5–20 ng/ml), and lowest (<1 ng/ml) at the beginning of the cycle, or in post-menopausal individuals (Tavita & Greaves, 2017). This hormone is highly protein bound and is routinely measured in blood to assess ovary function. Like estradiol and testosterone, immunoassays for progesterone quantification have been available since the 1970s but continue to suffer from limitations related to specificity and sensitivity (Kharma et al., 1972; O'Rorke, Kane, Gosling, Tallon, & Fottrell, 1994). For progesterone quantification, immunoassays can be affected by up to 10% cross-reactivity from steroids like 17α-hydroxyprogesterone and 11-deoxycorticosterone (Wilson et al., 1998) as well as contraceptive steroids (Kharma et al., 1972). As such, NIST approached the standardization of progesterone by the development of an LC–MS/MS method using isotope dilution, which was validated and published in 2006 (Tai et al., 2006). This method, one of the first in the literature capturing LC–MS/MS quantification of progesterone in serum, was validated against the reference GC–MS method using CRM 347 from the European Commission Institute for Reference Materials and Measurements (Tai et al., 2006; Thienpont, Siekmann, Lawson, Colinet, & De, 1991). The investigators at NIST have revealed the photosensitivity of progesterone, which must be considered during sample handling, which in their case includes a pH adjustment (pH 9.8) followed by hexane LLE (Tai et al., 2006). The m/z transition used for +ESI multiple reaction monitoring (MRM) (314.8 à 97.1) is the same as or similar to what is used in many other progester- one assays moving forward (Desai, Harwood, & Handelsman, 2019; Farke et al., 2011; Gaikwad, 2013; Galligan et al., 2018; Gao et al., 2015; Genangeli et al., 2017; Gibson, Bucknall, Golebiowski, & Stapleton, 2019; Gomez-Gomez et al., 2020; Häkkinen et al., 2018;

Higashi et al., 2011; Lee, Lee, Hong, Chung, & Choi, 2016; McCulloch & Robb, 2017; Olisov et al., 2019; Schiffer, Adaway, Baranowski, Arlt, & Keevil, 2018; Surowiec, Koc, Antti, Wikström, & Moritz, 2011; Tai et al., 2006; Voegel et al., 2018; Zhou & Cai, 2020).

5. Matrices And Sample Preparation

5.1. Testosterone analysis from serum and plasma

Of the methods surveyed for this review, the majority opted for the same (serum) or similar (plasma) matrix as the CDC and NIST RMPs. Sample volumes ranged from 100 to 500 µg/ml as summarized in Table 4.1. The lipophilicity of testosterone is conducive to sample preparation techniques that exploit this feature, such as LLE. LLE relies on the partition of the analyte from the aqueous matrix into an immiscible organic solvent. Methyl tert-butyl ether (MTBE) is the most common LLE solvent used to extract testosterone from serum (Handelsman, Desai, Seibel, Le Couteur, & Cumming, 2020; Keski-Rahkonen, Huhtinen, Poutanen, & Auriola, 2011; Keski-Rahkonen, Desai, Jimenez, Harwood, & Handelsman, 2015; Legacki et al., 2020; Matysik & Liebisch, 2017; Yuan et al., 2019), but is also applied to extractions from plasma (Desai et al., 2019). Other serum relevant sol- vents include toluene (Häkkinen et al., 2018), 1-chlorobutane (Ke, Bertin, Gonthier, Simard, & Labrie, 2014), dichloromethane (Qin et al., 2020) and di-isopropyl ether (Star-Weinstock, Williamson, Dey, Pillai, & Purkayastha, 2012). Of note, toluene has also been used as an extraction solvent for plasma preparation (Häkkinen et al., 2018) and is an important dopant in assays utilizing APPI as the mass spectro- metric source (McCulloch & Robb, 2017). The CDC assay utilizes a mixture of ethyl acetate and hexane as an extraction solvent (Botelho et al., 2013), a practice found in methods by other investigators quantifying testosterone from serum (Kaufmann et al., 2019; Schofield, Mendu, Ramanathan,

Pessin, & Carlow, 2017). Combining solvents for an LLE protocol can maximize the distribution ratio (KD), thus improving individual analyte recovery, or expand the suitability of the extraction to capture more analytes (Majors, 2008). The latter justification was probably sought by Kauffman and colleagues in their assay for testosterone, endogenous estrogens and over 35 other hormones from a variety of matrices, serum included (Kaufmann et al., 2019). Of note, these investigators also employed pH adjustment to facilitate analyte transition out of the aqueous (matrix) phase (Kaufmann et al., 2019). Laszlo and colleagues verified the potential for high recovery of testosterone, progesterone, androstenedione, cortisol and 11 synthetic progestins from plasma using the ethyl acetate–hexane combination, but opted for the high throughput offered by 96-well plate SPE (Laszlo et al., 2019).

The relative "cleanliness" of the plasma and serum matrices allows some investigators to rely on protein precipitation alone to prepare samples for LC–MS/MS. Not surprisingly, the most frequently used solvents include methanol (Broccardo et al., 2013; McCulloch & Robb, 2017; Xu et al., 2017) and acetonitrile (Subhash Chandra Bose et al., 2013; Genangeli et al., 2017). The relevance of this approach is emphasized by the fact that most testosterone in circulation is protein bound (Tai et al., 2007). Some investigators use protein precipitation as a pretreatment for SPE, as in the investigation of hormone profiles in free-roaming bottlenose dolphins (Galligan et al., 2018). When testosterone was among other free and sulfated hormones measured in a study involving obese girls, the selectivity of the sample preparation called for SPE, in this case Strata-X, a polymeric phase suited for a spectrum of acidic, basic and neutral analytes (Lee et al., 2016). Finally, the most unique approach for preparing plasma/serum sam- ples for testosterone analysis utilized solid–liquid extraction (SLE) + with dichloromethane elution

(Wooding et al., 2015). This technique operates on a principle similar to LLE but aims for additional interferant removal through interaction with the solid phase.

5.2. Other matrices used in testosterone quantification

Less invasive human matrices for testosterone quantification include fingernails (Higashi et al., 2016; Voegel et al., 2018), urine (Son, Yun, & Cho, 2020; Zheng et al., 2019; Zhou & Cai, 2020) and saliva (Gao et al., 2015; Thieme, Rautenberg, Grosse, & Schoenfelder, 2013). Utilization of fingernails as a matrix requires 0.5–10 mg material (Higashi et al., 2016; Voegel et al., 2018) and has the potential to provide a retrospective time window of hormone levels similar to hair. In an investigation of sex and handed differences, Higashi found detectable levels of testosterone in fingernails compared with more hydrophilic steroids (Higashi et al., 2016). This is probably due to its high affinity to keratin. Fingernail samples could be prepared using SPE (Higashi et al., 2016) or LLE (Voegel et al., 2018).

Despite the long-standing tradition of favoring urine as a matrix for anabolic steroid testing (Gosetti, Mazzucco, Gennaro, & Marengo, 2013), the assays found for testosterone quantification in urine are only from the last few years (Son et al., 2020; Zheng et al., 2019; Zhou & Cai, 2020). In the most comprehensive profiling assay found in our literature search, Son and colleagues quantified 39 steroids, including testosterone, from urine (Son et al., 2020). Pre-treatment with β-glucuronidase followed by SPE using an HLB cartridge enabled the investigators to capture total hormone concentrations (Son et al., 2020). Furthermore, mobile phase additive optimization revealed that 5 mM ammonium formate gave the highest signal for testosterone, yet this was not reflected in the final published assay (Son et al., 2020). A similar approach toward metabolomic profiling of steroid hormones in urine utilized β-glucuronidase and LLE to prepare urine from pregnant women (Zhou & Cai, 2020). Despite acceptable levels

of precision and accuracy for most analytes, these investigators cite the lack of matched internal standards for every analyte as limitation of their assay (Zhou & Cai, 2020). Furthermore, the lack of matched deuterium-labeled internal standards for every analyte could have contributed to some R^2 values of <0.99 (Zhou & Cai, 2020). By grouping the steroid hormones into three subclasses based on diagnostic product ions (DPIs), Zheng et al. characterized 80 different compounds in male and female urine (Zheng et al., 2019). In contrast to the other assays relying on a urine matrix, these samples were prepared using SPE (Waters Oasis HLB). The three subgroups included corticosteroids, progestogens and androgens with and without a C-11 substituent, and estrogens. MS^2 fragments of m/z 97.065, 109.065 and 123.080 served as DPIs for the non-C-11 substituted compounds, m/z 121.065 and 163.112 for those with a C-11 substituent, and m/z 107.049, 133.065 and 159.080 for estrogens (Zheng et al., 2019).

Investigators using saliva as a matrix for monitoring hormone concentrations following transdermal testosterone administration found concentrations to be more sensitive to dosing changes than urinary hormone levels (Thieme et al., 2013). This could be explained by a more direct relationship between the biologically active testosterone in circulation (unbound) and salivary concentrations, which are not predicated on renal excretion (Thieme et al., 2013). In this study, the saliva samples were prepared using an MTBE extraction from the collection swabs, prior to LC–MS analysis, while the comparator urine was prepared by the same LLE followed by silylation derivatization for GC–MS analysis. An additional advantage the investigators note for choosing saliva over urine for monitoring testosterone levels is the elimination of interference from compounds such as ethanol and 5αreductase inhibitors, which affect testosterone metabolism, but should not influence oral concentrations (Thieme et al., 2013). Other investigators verify the sensitivity of saliva for testosterone quantification, among other

hormones, cautioning against possible matrix signal enhancement and requiring correction using an internal standard (Gao et al., 2015). Of note, the Gao et al. assay uses in-line SPE for saliva sample preparation, allowing for higher sample throughput (5.20 minutes/sample) vs. methods requiring offline manipulation (Gao et al., 2015).

Less common matrices used for testosterone quantification include tears (Gibson et al., 2019), breast milk (Gomez-Gomez et al., 2020), amniotic fluid (Gomez-Gomez et al., 2020), endometrium (Häkkinen et al., 2018), breast tissue (Gaikwad, 2013) and H295R cells (Zhang et al., 2011). Because steroid hormone concentrations have been associated with dry eyes, Gibson and colleagues chose to investigate the possibility of using tears as a matrix. Despite the challenge of working with very small sample volumes, 14 steroid hormones could be simultaneously quantified using ±APCI-LC–MS/MS (Gibson et al., 2019). Additionally, these investigators examined three sample preparation approaches for tears, including protein precipitation (PP) with cold ethanol, LLE with MTBE–diethyl ether and Schirmer strips, finding that the LLE was the least acceptable in terms of analyte recovery (Gibson et al., 2019; Pieragostino et al., 2017). Testosterone was one of 28 hormone analytes investigated for quantification in amniotic fluid and one of 12 investigated in breast milk (Gomez-Gomez et al., 2020). These scientists divided their groups of analytes by type of parent MS ion formed. As such, testosterone, as well as 17β-estradiol and progesterone were characterized by their $[M + H]^+$ (Gomez-Gomez et al., 2020). These two matrices required different sample preparation approaches, PP (acetonitrile) for amniotic fluid and LLE (ethyl acetate) for breast milk. In this instance, salting out with NaCl was used to increase recovery of steroid hormones from the breast milk (Gomez-Gomez et al., 2020). Gaikwad's investigation of the steroid hormone metabolome offers the most comprehensive LC–MS/MS assay found in this review. In this

study, 101 compounds could be extracted from breast tissue using protein precipitation and ultrafiltration (Gaikwad, 2013). In an investigation of the link between endometriosis and steroid hormones, Häkkinen quantified testosterone, progesterone and 17β-estradiol, plus over 20 other analytes, in endometrial tissue (Häkkinen et al., 2018). In comparing MTBE and toluene as LLE solvents, as well as several SPE cartridges, these investigators cited MTBE's tendency to allow lipophilic interferants into the final extract while SPE extracts were laden with hydrophilic interferants (Häkkinen et al., 2018). H295R cells are used by the Environmental Protection Agency (EPA) for the assessment of endocrine disruptive compound exposure in the environment (Zhang et al., 2011). Cell cultures of this type respond similarly to LLE as plasma/serum with similar limits of quantification (10 pg/ml) for testosterone and 17β-estradiol as other matrices (Zhang et al., 2011).

5.3. Highly sensitive assays for determining 17-β- estradiol in serum and plasma

For clinical applications of estradiol quantification, plasma and serum are the most widely utilized matrix, as summarized in Table 1. A 2015 review of estradiol assays qualified the need for clinically relevant LC– MS/MS methods capable of accurately quantifying concentrations of 0.2 pg/ml (Ketha et al., 2015). For the methods surveyed for this review, very few provided low picogram-level lower limits of quantification (LLOQ) for 17-β-estradiol (Beinhauer et al., 2015; Denver et al., 2019; Dowis, Woroniecki, & French, 2019; Faqehi et al., 2016; Ke et al., 2014; Li & Franke, 2015; Ray, Kushnir, Bunker, Rockwood, & Meikle, 2012; Schofield et al., 2017; Wang et al., 2016). Many of the highest sensitivity assays for estradiol quantification require analyte derivatization, discussed in Section 6 of this review. For example, using the combination of a bulk derivatization scheme and an on- column protein trap, Beinhauer and colleagues achieved an LLOQ of 5 pg/ml for several estrogens in bovine serum (Beinhauer

et al., 2015). These investigators use the term “bulk derivatization” to describe applying a single derivatizing agent, in this case 2-fluoro- 1-methyl-pyridium p-toluenesulfonate (2-FMP), to a spectrum of analytes, effectively creating dansylated derivatives prior to additional cleanup (Beinhauer et al., 2015). In an investigation of the effects on estrogen metabolism associated with polyaromatic hydrocarbon (PAH) exposure, a derivatization approach utilizing quaternization of the analytes allowed investigators to achieve 1 pg/ml quantification of 17-β-estradiol (Denver et al., 2019). The approach of using derivatizing agents capable of quaternization had been applied earlier to the investigation of six free and conjugated estrogens in serum from post-menopausal women achieving an even lower LLOQ of 0.5 pg/ml (Wang et al., 2016). Other investigators of serum hormones in post-menopausal women utilized dansyl chloride to specifically derivatize 17-β-estradiol and estrone while sparing other hormones in the assay (Ke et al., 2014). This derivatization approach was also applied to an estradiol in serum assay, where the investigators vali- dated their approach against the CDC RMP, achieving an LLOQ of 2 ng/ml (Dowis et al., 2019). One of the lowest quantification limits reported utilized four different derivatizing agents for preparation of serum samples to maximize sensitivity and specificity for measuring estrogens plus several metabolites from human serum (Li & Franke, 2015). Schofield and colleagues present one of the few low- pg/ml assays for 17-β-estradiol that does not involve derivatization (Schofield et al., 2017). In this method, LLE using hexane–ethyl acetate (9:1), which was previously optimized for testosterone by the CDC HoST program, also adequately extracts estradiol from the serum matrix prior to LC–MS/MS using −ESI (Botelho et al., 2013; Schofield et al., 2017).

5.4. Alternative matrices for 17-β estradiol quantification

Estradiol was one of over 35 analytes included in steroidomic profiling assays from bovine urine (Kaabia et al., 2018; Kaufmann et al., 2019). These methods differed in their approach for sample preparation including SPE (C_{18} and HLB) to capture free and conjugated steroids (Kaabia et al., 2018) vs. LLE preceded by β-glucuronidase treatment to promote formation of unconjugated analytes (Kaufmann et al., 2019). Similarly, assays for steroid profiling using human urine utilize SPE pretreatment (Son et al., 2020; Zheng et al., 2019) or LLE (Zhou & Cai, 2020). An assay more focused on estrogen profiling in human urine was applied to evaluating post-menopausal women for breast cancer risk (Xu et al., 2005). While post-menopausal serum sampling requires pg/ml level sensitivity, concentrations in urine are higher; thus, the mid-ng/ml LLOQ in this method (Xu et al., 2005). Other noninvasive matrices for 17-β-estradiol quantification include saliva (Gao et al., 2015) and tears (Gibson et al., 2019). With a sample size of only 100 µl saliva, Gao and colleagues were also able to separate 17-βestradiol from six other analytes, including progesterone and testosterone (Gao et al., 2015). The combination of in-line SPE reduced sample handling and allowed for high-throughput quantification and eventual application to a re-analysis of nonsensical immunoassay results (Gao et al., 2015). As seen in Figure 4.3, this method shows one of the shortest, yet baseline resolved, chromatographic separations of estradiol, progesterone and testosterone (Gao et al., 2015). For the determination of several sex hormones in human tears, estradiol had the highest LOD and LOQ, limiting the investigators' ability to compare tear levels with serum estradiol (Gibson et al., 2019). Of the three sample preparation techniques evaluated by this team, PP with cold ethanol yielded the highest extraction recovery for estradiol (Gibson et al., 2019).

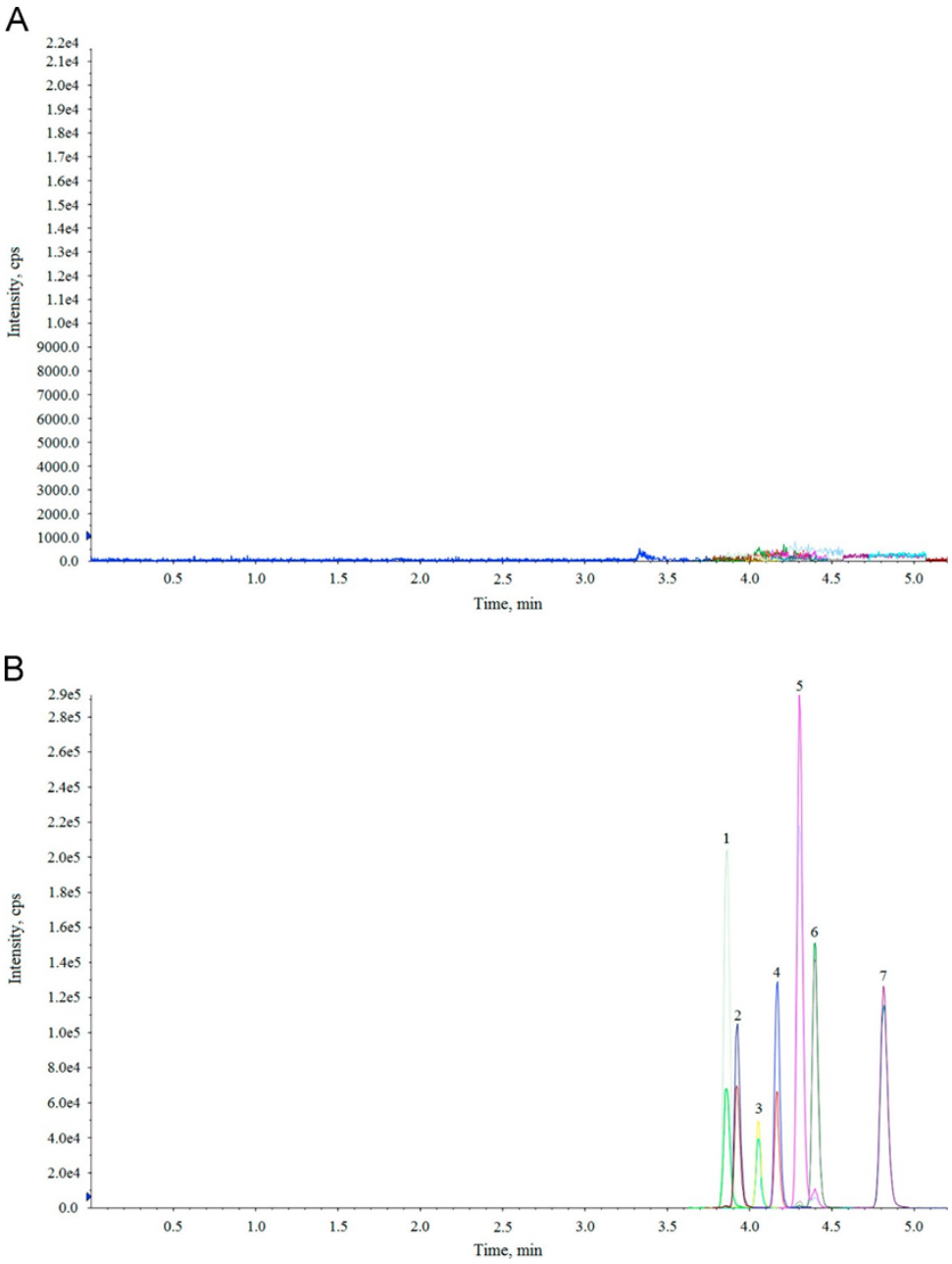

Figure 4.3: Chromatogram showing resolution of testosterone (five), progesterone (seven) and 17-β estradiol (four) in blank saliva (a) and spiked blank saliva (b). Reprinted (figure 1) with permission from Gao, W., Stalder, T., & Kirschbaum, C. (2015). Quantitative analysis of estradiol and six other steroid hormones in human saliva using a high throughput liquid chromatography–tandem mass spectrometry assay. Talanta, 143, 353–358. https://doi.org/10. 1016/j.talanta.2015.05.004

Several research groups developed LC–MS/MS methods to quantify 17-β-estradiol in matrices related to its role as a female sex hormone. Estradiol was among 11 analytes validated in the LC–MS/MS quantification from human plasma, serum, endometrium and endometriosis tissue (Häkkinen et al., 2018). Short-term stability tests indicated that estradiol concentrations in the tissues could decrease by up to 13% from baseline if left at room temperature for 1 h; therefore, investigators recommended immediate cold homogenization and analysis (Häkkinen et al., 2018). The reported sensitivity for estradiol in this method was relevant to levels found in premenopausal women (Tosti et al., 2017). LC–MS/MS of androgens and estrogens from ovarian follicular fluid provides a unique approach to understanding a patient's response to fertility assistance, because higher concentrations of estradiol in this matrix are associated with functional oocytes (Kushnir et al., 2009). Liquid–liquid extraction with MTBE followed by derivatization with dansyl chloride was used to prepare the samples, enabling positive-mode TurboIonSpray (Kushnir et al., 2008). The data was expressed as estradiol–testosterone ratios (Kushnir et al., 2009). Estradiol is among over 100 endogenous and exogenous steroid hormones included in a breast tissue steroidomic profiling assay published in 2013 (Gaikwad, 2013). This ultra-high-performance (UHPLC)- MS/MS method employs a unique column type (HSS T3) to provide some aspect of separation to the large analyte pool in only 12 min. Furthermore, it allows for the comprehensive evaluation of estrogen metabolites in breast tissue, opening the door for biomarker discovery (Gaikwad, 2013). An estrogen-focused assay for comparing the differences in normal and breast cancer-positive breast tissue samples utilized SPE (C8) plus a unique 3-bromomethyl-propyphenazone (BMP) derivatization (Khedr & Alahdal, 2016). This approach not only increased sensitivity vs. using dansyl chloride (DC), but also made the

samples stable enough to withstand three freeze–thaw cycles and up to 30 days of storage (Khedr & Alahdal, 2016).

5.5. Saliva and other matrices for progesterone quantification

While plasma and serum prevail as the most common matrices for progesterone assays that utilize LC–MS/MS technology (see Table 4.1), saliva is also a popular choice given the noninvasive nature of its collection (Gao et al., 2015; Gomez-Gomez et al., 2020; 21, Schiffer et al., 2018). Furthermore, many of the clinical applications of progesterone monitoring require repeat sampling, thus making this noninvasive quality that much more desirable. A 2015 method published by Gao focused on estradiol quantification in saliva, but also included other hormones, such as progesterone and testosterone (Gao et al., 2015). Despite the sample volume of 100 μl, these investigators successfully employed online SPE to achieve an LLOQ of 0.01 ng/ml progesterone, sufficient for the clinical demands of progesterone monitoring (Gao et al., 2015). One limitation of this method may be the use of the Salivette device for collection, which has been shown to skew quantification of salivary hormones owing to nonspecific binding (Gröschl, Köhler, Topf, Rupprecht, & Rauh, 2008). Saliva was included, along with amniotic fluid and breast milk, in an assay for investigating endogenous hormone levels of over 30 analytes, including progesterone (Gomez-Gomez et al., 2020). These investigators demonstrated that more polar steroids could not be detected in saliva, while progesterone was the longest retained analyte in the saliva profile (Gomez-Gomez et al., 2020). For assays that considered salivary progesterone alone, run times were short (5–7 min), and matrix effects were limited (Higashi et al., 2011; Schiffer et al., 2018). Furthermore, isocratic elution could even be used in the chromatographic separation, as well as sample volumes as low as 50 μl (Schiffer et al., 2018). These investigators demonstrated that both LLE, using dichloromethane (Schiffer et al., 2018)

and SPE, using Strata X cartridges (Higashi et al., 2011), are suitable for preparing saliva samples for LC–MS/MS. Finally, the use of male saliva proved to be a suitable blank in the investigation of salivary and serum concentrations of progesterone in pregnant and nonpregnant women (Higashi et al., 2011).

Zhang and colleagues developed an LC–MS/MS approach to quantify progesterone in plasma, in the presence of 17α- hydroxyprogesterone, as this steroid is known to interfere with the immunoassay quantification of progesterone (Wilson et al., 1998; Zhang et al., 2008). Using a reversed-phase gradient separation, the progesterone was chromatographically resolved from this interferant, and resolved using mass transitions. Limiting the analyte targets allowed these investigators to keep a short run time (7 min) and even demonstrate acceptable precision and accuracy without the use of a stable isotope internal standard (Zhang et al., 2008). For methods related to progesterone from serum or plasma, many include this hormone among a panel of others, including testosterone and 17β-estradiol, and have already been discussed in previous sections.

Progesterone and other lipophilic steroid hormones are suitable for quantification in many tissue matrices. In a novel assay quantifying progesterone and other steroid in fingernails, Voegel and colleagues found no handed differences in progesterone accumulation in nails (Voegel et al., 2018). A quantification of progesterone, testosterone and hydrocortisone in whale blubber boasted smaller sample sizes vs. what is required for immunoassay (Hayden et al., 2017). Despite the complexity of the matrix, protein precipitation coupled with LLE was sufficient sample cleanup to achieve acceptable precision and accuracy with low-nM (fg/µl) LLOQs. This was facilitated by nanoLC in the chromatographic separation (Hayden et al., 2017). In a study of 11 steroid hormones in mouse tissues, including prostate, ovary, testi- cle and

adrenal gland, matrix effects from the tissue could be mitigated by the addition of SPE to sample preparation for early-eluting, more polar analytes, but progesterone recovery was negatively affected by the application of HLB SPE cartridges (Surowiec et al., 2011). The authors speculate that the use of the 5% MeOH wash step is more effective at removing polar interferants, but less impactful for recovery of compounds like progesterone, dihydroxytestosterone and dehydroepiandrosterone (DHEA) (Surowiec et al., 2011). Despite this, several other investigators found HLB-phase SPE columns to be beneficial for progesterone extraction in other matrices, including urine (Son et al., 2020; Zheng et al., 2019) and plasma (Zhang et al., 2008). For more complex matrices, the absence of SPE meant zero analyte recovery for some steroids, but it did not benefit all analytes equally (Surowiec et al., 2011).

6. The Role Of Derivatization In Sex Steroid LC–MS

6.1. Estradiol

The role of derivatizing 17-β-estradiol during sample preparation for LC–MS/MS analysis from a biological matrix cannot be overstated. In fact, most of the low-pg/ml assays for this hormone employed some derivatization scheme as an add-on to LLE or SPE (Beinhauer et al., 2015; Denver et al., 2019; Faqehi et al., 2016; Ke et al., 2014; Li & Franke, 2015; Ray et al., 2012; Wang et al., 2016). Derivatization of these steroid hormones prior to quantification by LC–MS/MS is reported to increase ionization efficiency (Li & Franke, 2015), and is considered by many to be necessary to achieve low pg/ml LLOQ targets (Santa, 2013). Furthermore, derivatization often enables the use of positive ionization mode for mass spectrometric detection while underivatized estradiol is typically run in negative mode (Cohen, Ross, Smith, & Fawcett, 2017; Farke et al., 2011; Häkkinen et al., 2018; Schofield et al., 2017). A variety of derivatizing

agents are available to help increase the ionizability of 17-β-estradiol and other estrogens. Often, they do not react with other hormones in the assay, unless the structures contain a 3-OH on ring A of the steroid backbone (Keski-Rahkonen et al., 2015). Common derivatization agents for 17-β-estradiol include DC, 2-FMP, 1,2-dimethylimidazole-5-sulfonyl chloride (DMIS), and N-methyl pyridinium-3-sulfonyl (NMPS). When estrogen profiling experiments are conducted using Orbitrap technology, a combination of four derivatizing agents can be used in the sample preparation to confer an array of specificity (Li & Franke, 2015). Researchers who opt to forego derivatization of estradiol cite the possibility of isomer formation, varying rates of reaction and forming side products (Häkkinen et al., 2019). On the other hand, the absence of derivatization for a wide variety of steroid hormone analytes requires polarity switching, which may compromise overall sensitivity (Fülöp, Vari, Miklos, & Imre, 2017).

Dansyl chloride (DC) is the most commonly used derivatization method for estradiol (Anari et al., 2002; Beinhauer et al., 2015; Dowis et al., 2019). DC enhances the ionization of phenolic hydroxyl group- containing estrogens by adding an amine group to the 17-β-estradiol molecule, shifting the predicted pKa to 3.3 ± 0.4, as shown in Figure 4.4 (Anari et al., 2002; Bichon et al., 2012; Qin et al., 2020). DC enhances the LLOQ of 17-β-estradiol measurement by introducing a charged group on the molecule, further enhancing ionization efficiency (Dowis et al., 2019; Ke et al., 2014; Kushnir et al., 2011; Nair, Patel, Sanyal, Singhal, & Shrivastav, 2018). DC also increases the stability of the 17-β-estradiol in solution so it is stable for several days when refrigerated (Bichon et al., 2012; Ke et al., 2014; Nair et al., 2018). DC can be used in conjunction with other derivatization agents to simultaneously quantify 17-β-estradiol and other compounds. Various studies have reported different optimal conditions for the use of DC for estradiol derivatization including optimal temperature, time and pH. However, the reaction is

facilitated by basic conditions and mild heating (60 °C). One downside to the use of DC for derivatization is the possibility of false results owing to the fact many endogenous substances could readily react with DC. The issue can be partially addressed by using charcoal stripping as part of sample preparation (Nair et al., 2018). However, charcoal stripping is time consuming, can interfere with method validation and may remove matrix components and compromise the relevance of the matrix (Cao et al., 2009; Dang & Lowik, 2005). Finally, DC derivatization is often coupled with an extraction step and seems suited to follow SPE (Galligan et al., 2018; Nair et al., 2018), LLE using 1-chlorobutane (Ke et al., 2014), MTBE (Kushnir et al., 2009; Ray et al., 2012; Yuan et al., 2019), hexane–ethyl acetate (Dowis et al., 2019) or dichloromethane (Qin et al., 2020; Xu et al., 2005; Xu et al., 2007; Zhang et al., 2011).

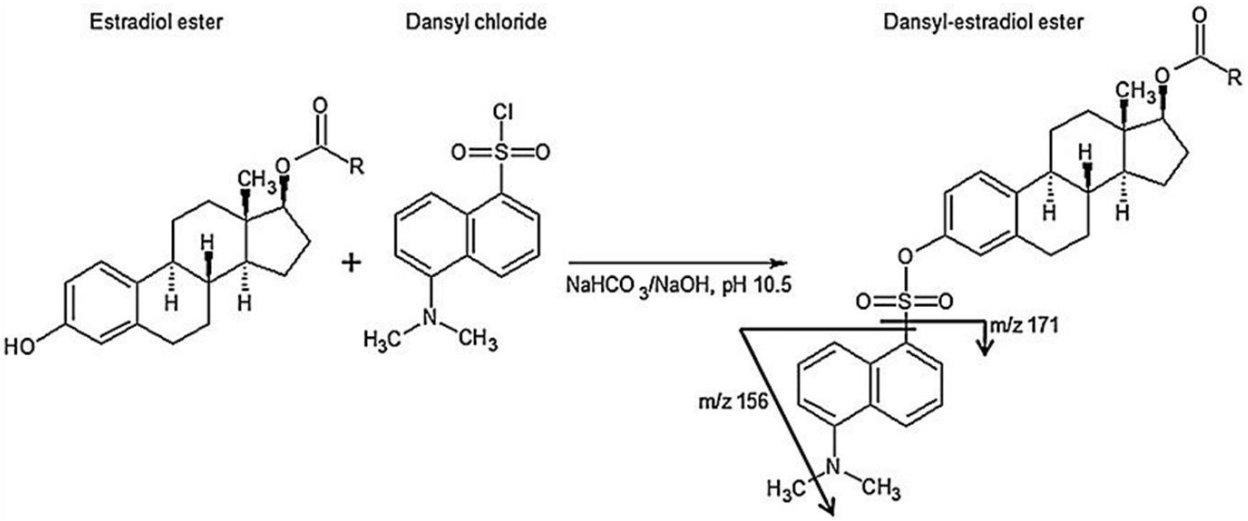

Figure 4.4: Formation of dansyl chloride derivative on estradiol. Reprinted (figure 2) with permission from Bichon, E., Béasse, A., Prevost, S., Christien, S., Courant, F., Monteau, F., & Bizec, B. L. (2012). Improvement of estradiol esters monitoring in bovine hair by dansylation and liquid chromatography/tandem mass spectrometry analysis in multiple reaction monitoring and precursor ion scan modes. Rapid Communications in Mass Spectrometry, 26(7), 819–827. https://doi.org/10.1002/rcm.6160

To bypass the need for charcoal stripping (Nair et al., 2018) and to improve specificity for estrogens in bioanalysis (Faqehi et al., 2016), 2-FMP provides a viable alternative to the more widely used DC. Investigators using 2-FMP prefer some cation exchange treat- ment of the sample before (Faqehi et al., 2016) or after derivatization (Beinhauer et al., 2015). Beinhauer and colleagues coupled bulk derivatization with weak cation exchange restricted-access media-based trap-and-elute LC–MS/MS by applying the 2-FMP derivatization to a small (100 μl) serum sample (Beinhauer et al., 2015). This reduced handling of the sample and time of analysis facilitates high sensitivity quantification (Beinhauer et al., 2015). 2-FMP has also been reported to make the reaction more specific than other derivatization methods, including dansylation (Faqehi et al., 2016). Unlike DC, 2-FMP does not stabilize samples in the refrigerator for days; therefore, they must be analyzed within 8 h. They were reported to be stable for up to 48 h when frozen at −80°C (Beinhauer et al., 2015; Faqehi et al., 2016). Of note, 17-β-estradiol has been shown to be more affected by freeze– thaw cycles than other steroid sex hormones in biological samples (Handelsman et al., 2020). In addition, awareness of sample stability is paramount in this already challenging bioanalytical landscape.

Derivatization of steroid mixtures with DMIS shows high specificity to 17-β-estradiol as indicated in the mass spectral fragments in Figure 4.5 (Keski-Rahkonen et al., 2015). Introducing DMIS derivatization resulted in a 10-fold increase in sensitivity compared with a previously published method to measure 17-β-estradiol without derivatization (Harwood & Handelsman, 2009; Keski-Rahkonen et al., 2015). While a basic pH solution was necessary for DMIS derivatization, this reaction is less affected by reaction temperature and time compared with DC (Keski-Rahkonen et al., 2015). Importantly, steroids without phenolic hydroxyl groups are not affected by DMIS, allowing for the simultaneous quantification of estradiol and underivatized

androgens. Furthermore, the specificity of the DMIS derivatization for estradiol facilitated a 10-fold increase in sensitivity compared with other methods, allowing for quantification in the 0.5–5 pg/ml range, relevant to children, males and post-menopausal females (Keski-Rahkonen et al., 2015).

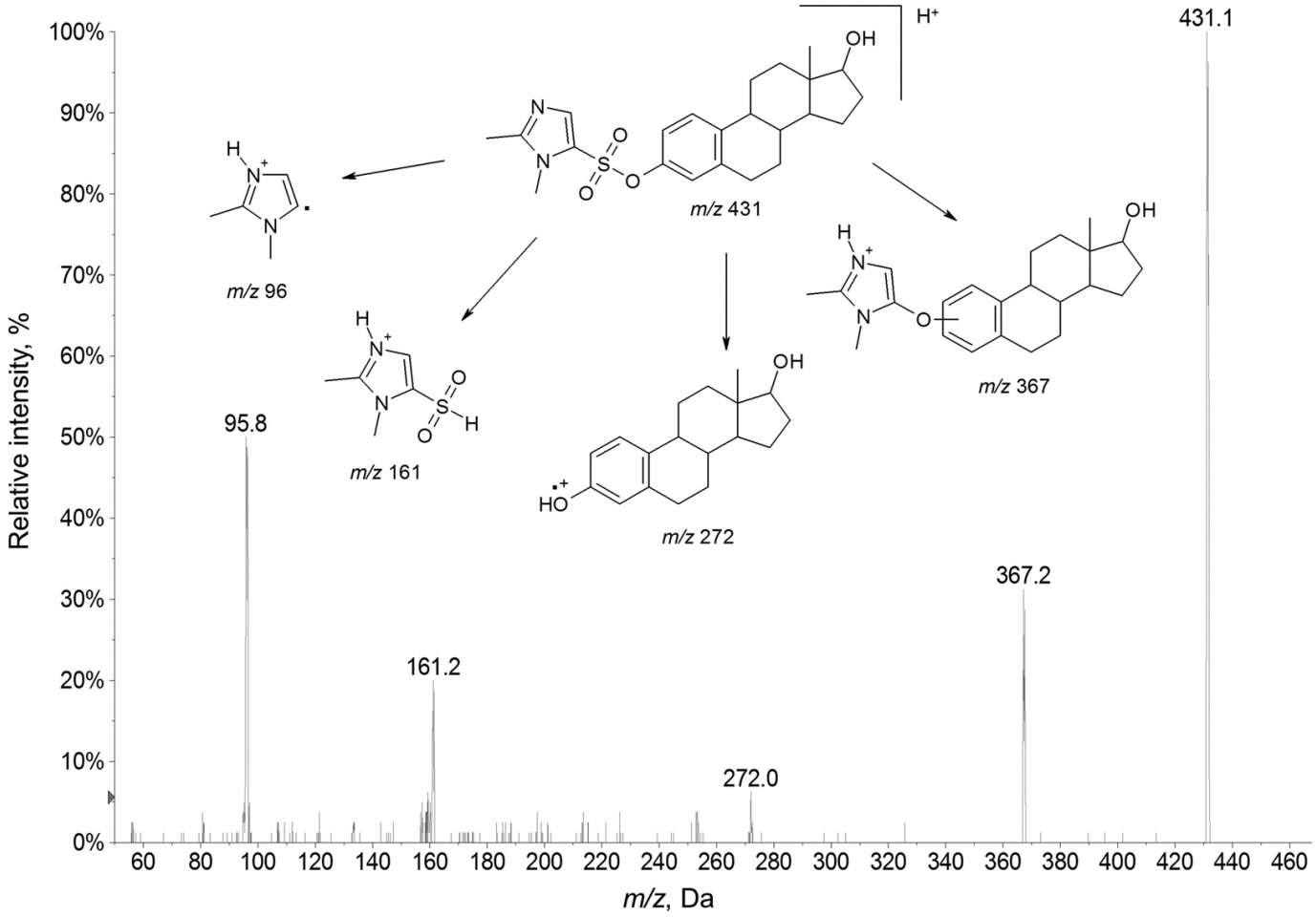

Figure 4.5: Product ion spectrum of 1,2-dimethylimidazole-5-sulfonyl chloride derivatized estradiol. Reprinted (figure 2) with permission from Keski-Rahkonen, P., Desai, R., Jimenez, M., Harwood, D. T., & Handelsman, D. J. (2015). Measurement of estradiol in human serum by LC–MS/MS using a novel estrogen-specific Derivatization reagent. Analytical Chemistry, 87(14), 7,180–7,186. https://doi.org/10.1021/acs. analchem.5b01042

An alternative approach to conventional derivatization methods involves the use of pre-ionized derivatives that eliminate the need for the protonation of the estradiol derivative. Pre-

ionized NMPS has been used to improve the sensitivity of 17-β -estradiol quantification by adding a Girard P derivative to the 17-oxo moiety of estradiol (Wang et al., 2016; Xu & Spink, 2008). This means that estrogen metabolites that do not contain the 17-oxo moiety cannot be quantified via this method, but excellent sensitivity can be achieved for 17-β -estradiol (Wang et al., 2016). Similarly, formation of methylpiperazine derivatives, as seen in Figure 4.6, results in quaternized analytes highly suited to positive-mode mass spectrometric detection (Denver et al., 2019).

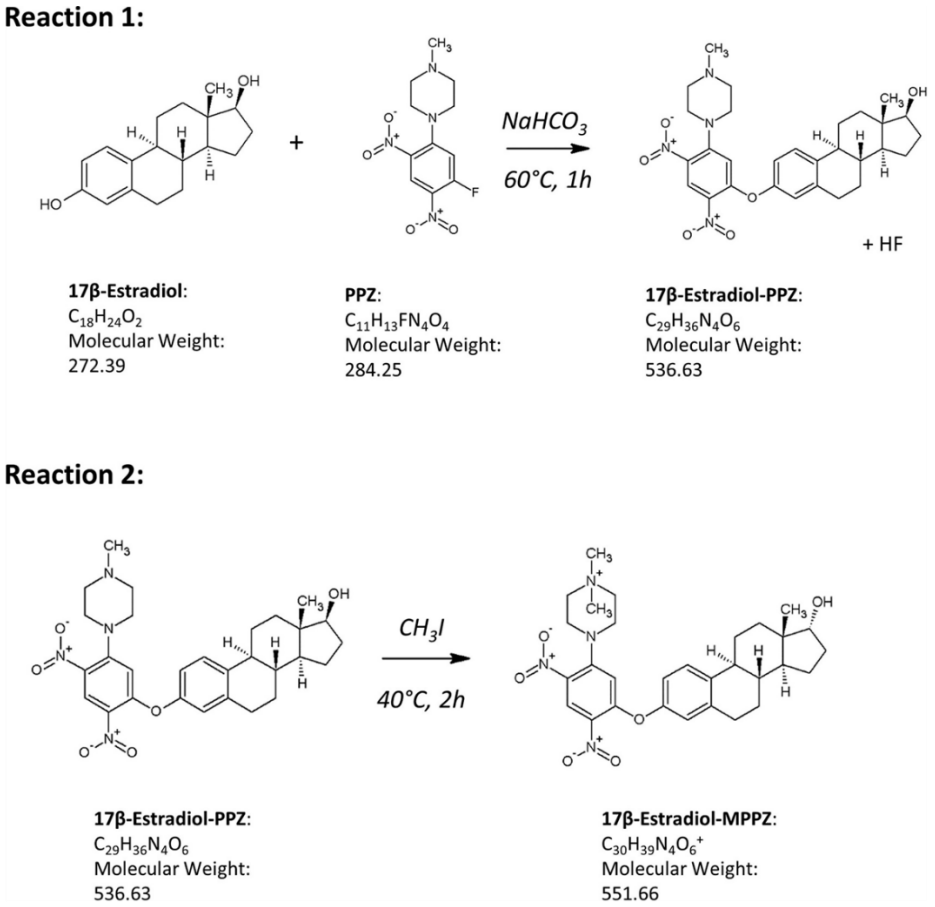

Figure 4.6: Formation of the 4-methylpiperazine derivative of 17β estradiol. Reprinted (figure 2) with permission from Denver, N., Khan, S., Stasinopoulos, I., Church, C., Homer, N. ZM., MacLean, M. R., & Andrew, R. (2019). Derivatization enhances analysis of estrogens and their bioactive metabolites in human plasma by liquid chromatography tandem mass spectrometry. Analytica Chimica Acta, 1,054, 84–94. https://doi.org/10.1016/j.aca.2018.12.023

6.2 Testosterone and progesterone

While mass spectrometric detection is significantly enhanced by derivatization of 17β-estradiol, this practice is less common for testosterone and progesterone assays. One exception is the use of O-(3-trimethylammoniumpropyl) hydroxylamine (QAO) to derivatize the ketone groups of testosterone and other ketosteroids (Häkkinen et al., 2019; Star-Weinstock et al., 2012). In this assay, LLOQ of 1 pg/ml was achieved in serum and dried blood spots (DBS), by

monitoring the quaternary aminooxy derivative of testosterone (Star-Weinstock et al., 2012). Furthermore, this enables for- mation of a unique fragment (m/z 344.3) following a neutral loss of (CH3)3N, as seen in Figure 4.7 (Star-Weinstock et al., 2012). Use of the QAO derivative offers the most sensitive, and one of the most rapid (3 min run time), assay possibilities for testosterone from a biological matrix (Star-Weinstock et al., 2012). Hydroxyl- amine derivatization was used to enhance the sensitivity of testosterone, progesterone and other hormone analysis from serum (Keski-Rahkonen et al., 2011). This derivatization is carried out in the final autosampler vial, requiring a 30 min heating step in the vial prior to injection (Keski-Rahkonen et al., 2011). Similarly, methoxyamine was used in one step of a two-phase derivatization protocol covering 29 different steroid hormones (Qin et al., 2020). This agent reacts with carbonyl groups present on structures like testosterone and progesterone while hydroxyl-containing steroids like 17β-estradiol can be covered by dansyl chloride derivatization (Qin et al., 2020). This strategy of splitting and recombining derivatized samples to cover a wide range of steroid hormones for LC–MS/MS analysis is seen in Figure 4.8 (Qin et al., 2020).

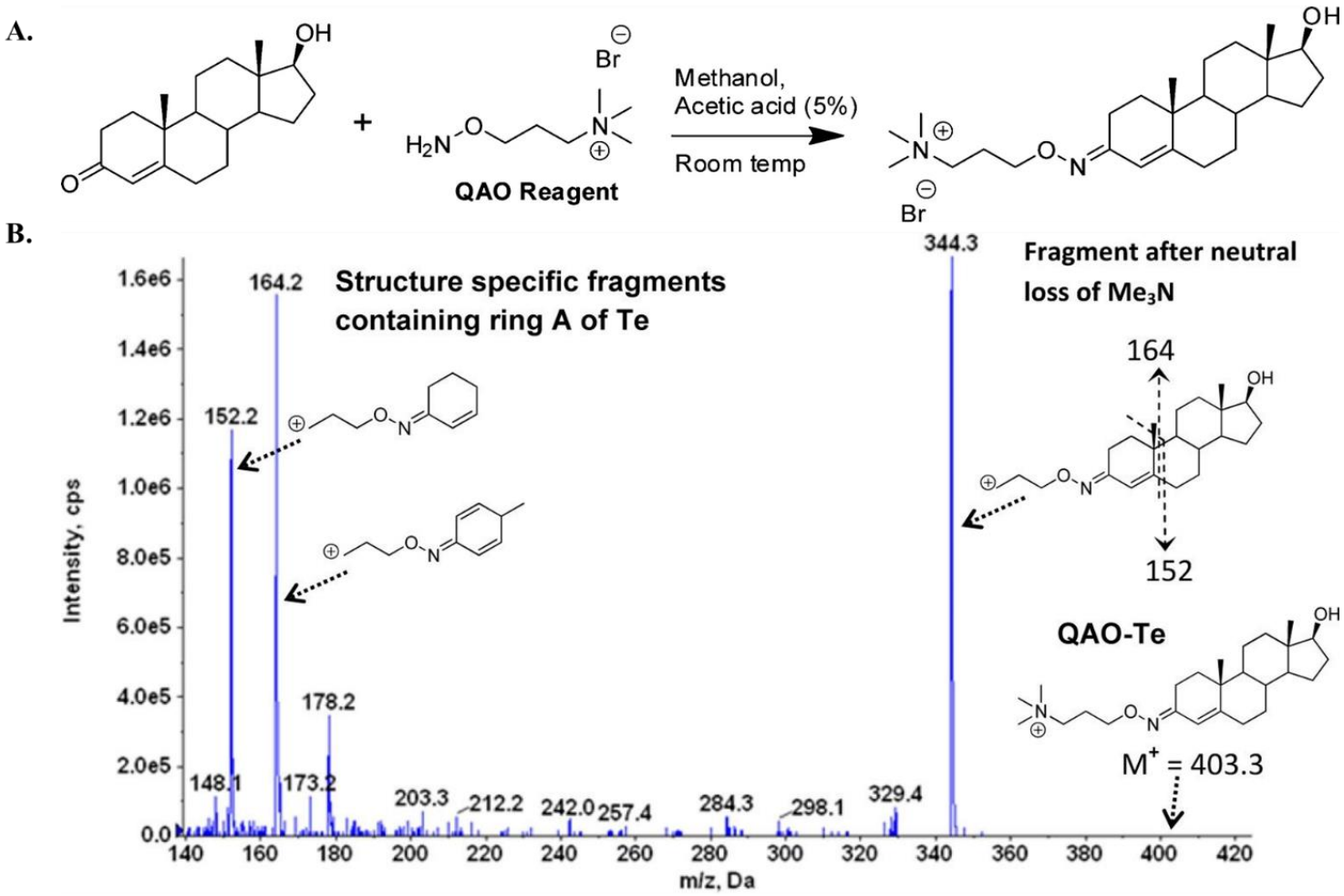

Figure 4.7: (**A**) Formation of the quaternary aminooxy derivative of testosterone. (**B**) Proposed + electrospray (ESI) fragmentation of quaternary aminooxy derivative of testosterone. Reprinted (figures 1 and 2) with permission from Star-Weinstock, M., Williamson, B. L., Dey, S., Pillai, S., & Purkayastha, S. (2012). LC–ESI–MS/MS analysis of testosterone at sub-picogram levels using a novel derivatization reagent. Analytical Chemistry, 84(21), 9,310–9,317. https://doi.org/10.1021/ac302036r

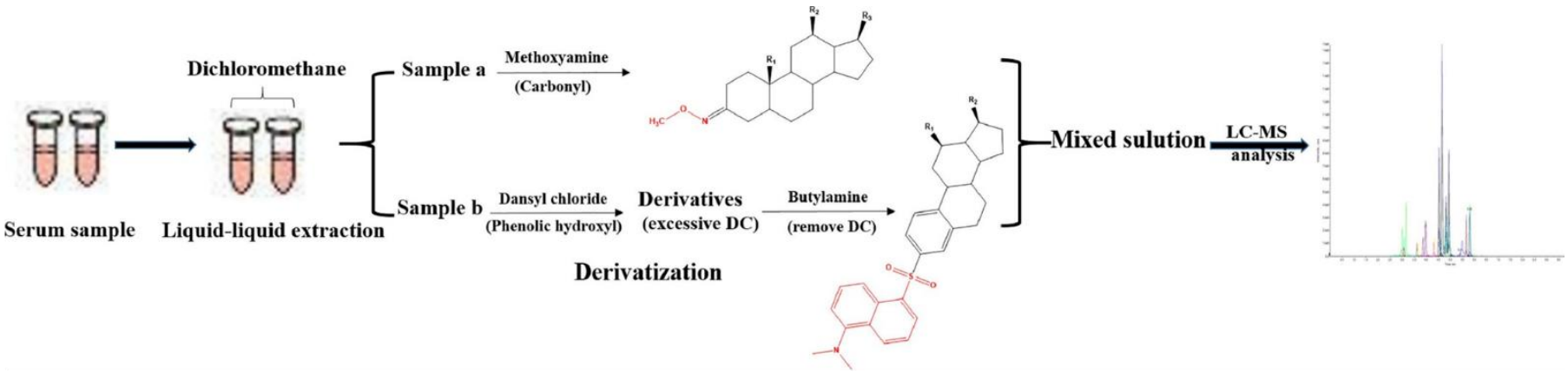

Figure 4.8: Parallel derivatization scheme for multicomponent steroid hormone assay. Reprinted (figure 1) with permission from Qin, Q., Feng, D., Hu,C.,Wang, B.,Chang, M., Liu, X., Yin, P., Shi, X., & Xu, G. (2020). Parallel derivatization strategy coupled with liquid chromatography–mass spectrometry for broad coverage of steroid hormones.Journal of Chromatography A, 1614, 460,709. https://doi.org/10.1016/j.chroma.2019.460709

7. Chromatography Considerations for Quantification Of Steroid Sex Hormones By LC–MS

Chromatographic separation of steroid hormones is challenging owing to structural similarities among the compounds, their metabolites and their exogenous counterparts. Choi notes that a quality chromatographic separation will improve mass spectrometric sensitivity as well as avoid incorrect estimation of individual steroids (Choi, 2018).

7.1 Column choice and instrumental configuration

The lipophilicity of testosterone and progesterone makes them well suited for chromatographic conditions that include C_{18} or C_8 alkyl- based separations (see Table 4.1). The second most popular column choice is the biphenyl column (Desai et al., 2019; Galligan et al., 2018; Häkkinen et al., 2018; Matysik & Liebisch, 2017). The potential to add π–π interactions to facilitate separations makes biphenyl a reasonable choice in steroid hormone chromatography (Bell, Shollenberger, & Cramer, 2017). This column type was successfully applied to a highly inclusive biosynthetic and metabolic 18 analyte screening procedure for steroid hormones from human serum (Desai et al., 2019). Galligan demonstrated that biphenyl column was preferred over C_{18} for separation of testosterone, progesterone and 17β-estradiol from dolphin plasma or serum (Galligan et al., 2018). Similarly, a phenyl–hexyl column demonstrated baseline separation of testosterone from dihydrotestosterone and 17β-estradiol (Keski-Rahkonen et al., 2015). In the most inclusive assay surveyed for this review, Gaikwad used the HSS T3 column to invoke separation of parent and metabolite steroid hormones, because metabolites are typically more polar in nature (Gaikwad, 2013). Finally, the RP-Amide column presents an unusual choice for steroid hormone separation, but was used in a study for quantifying testosterone, 17β-estradiol and DHEA from adipose tissue (Yoo & Napoli, 2019). While the authors do not

specifically discuss their column choice, the alternative selectivity, compared with C_{18} or C_8, as well as improved resolution from matrix interferants, could have played a role in this column choice. RP-Amide columns consist of a stable amide group embedded in a C_{18} chain, and have retention properties governed by Van der Waals forces and dipole forces, unlike C_{18} columns that are restricted to Van der Waals (Liu, Tanaka, Yamauchi, Testa, & Chuman, 2004). Of the assays surveyed for this review, over 35% utilize a sub-2 μm particle size column (see Table 4.1). This demonstrates the growing reach of ultra-high-performance liquid chromatography (UHPLC). The smallest particle size separation was accomplished on a microfluidic device (Waters nano Acquity UPLC Trizaic nanotile) for testosterone, progesterone and three other steroids (Broccardo et al., 2013). These devices profess over 100× gains in sensitivity and solvent conservation (Broccardo et al., 2013). As such, this method achieved LLOQs of <1 ng/mL and consumed only 3.06 microliters/min solvent (Broccardo, 2013).

A unique approach to improving estradiol resolution from interfering compounds in plasma involved linking two separation columns in tandem. As shown in Figure 4.9, the addition of a 2.1 × 30 mm Poroshell 120 SB-C_{18} column (2.7 μm) in front of the Zorbax SB-C_{18} Rapid Resolution HD column (2.1 × 50 mm, 1.8 μm) improved separation in unstripped serum vs. the Zorbax column alone (Ke et al., 2014). A 2D approach was also used by Mezzullo and colleagues where the sample was injected onto a POROS R1/20 column and back-flushed onto a Gemini C6-Phenyl column (2.0 × 100 mm, 3 μm) (Mezzullo et al., 2020). These conditions were optimized to prevent co-elution between estradiol and estrone as well as their respective 13C-labeled internal standards (Mezzullo et al., 2020). The 2D-LC approach is especially impactful when working with underivatized estradiol. This was demonstrated by using two Supelco LC-8-DB columns in tandem for estrone and estradiol quantification in serum (Fiers et al., 2012). This

allowed for the injection of a sample reconstituted in a 100% organic solvent without negatively affecting resolution (Fiers et al., 2012).

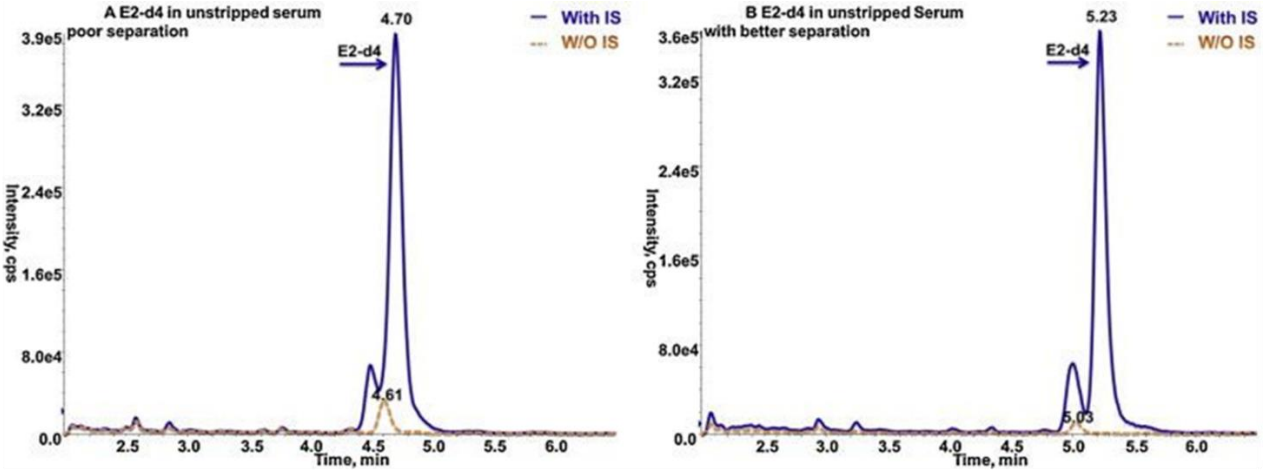

Figure 4.9: Improved resolution for estradiol achieved from 2D-LC separation. Reprinted (figure 2) with permission from Ke, Y., Bertin, J., Gonthier, R., Simard, J.-N., & Labrie, F. (2014). A sensitive, simple and robust LC–MS/MS method for the simultaneous quantification of seven androgen- and estrogen-related steroids in post-menopausal serum. The Journal of Steroid Biochemistry and Molecular Biology, 144, 523–534. https://doi.org/10.1016/j.jsbmb.2014.08.015

7.2 Mobile phase and separation conditions

Chromatographic mobile phase and delivery (isocratic vs. gradient) is a key feature of any separation. Not surprisingly, most of the assays surveyed here utilize a gradient separation. Of those running under isocratic conditions, targeting a single analyte (Higashi et al., 2011; Hogg et al., 2005; Ray et al., 2012; Tai et al., 2007) or restricting to a small number of analytes (Subhash Chandra Bose et al., 2013) makes this separation mode attainable. Ke and colleagues were able to separate seven androgen- and estrogen-related steroids in human serum under isocratic conditions, attributing this to the enhanced resolution inherent in a UHPLC column (Ke et al., 2014). Also running a seven-analyte steroid panel, McCulloch & Robb rely more on mass

separation through the triple quadrupole mass spectrometer than on chromatographic separation to achieve high-throughput analysis of steroids from serum, plasma and urine (McCulloch & Robb, 2017).

Mobile phase composition options include many common additives such as formic acid, ammonium formate, acetic acid and ammonium acetate, as summarized in Table 4.1. Selection of proper mobile phase additives is especially important in assays for steroid hormones because these compounds are plagued by low heteroatom account, limited fragmentation and overall low ionization efficiency (Kaufmann et al., 2019). The inclusion of ammonium hydroxide in an assay for estrone, 17β-estradiol and testosterone was more about preserving the column integrity and less related to analyte separation (Wooding et al., 2015).

Ammonium fluoride (NH4 F) as a mobile phase additive is seen in several steroid hormone assays, especially when both positive and negative ionizations modes are utilized (Häkkinen et al., 2018; Kaufmann et al., 2019; Schofield et al., 2017; Voegel et al., 2018). This additive is known to stabilize positive mode signals while enhancing the sensitivity of negative mode signals (Kaufmann et al., 2019; Li, Li, & Kellermann, 2018). The effect on signal enhancement for various estrogens is demonstrated in Figure 4.10 (Li et al., 2018). Relevant concentrations range from 0.2 to 100 mM NH_4F, yet higher concentrations (>1 mM) have been linked to pressure sensor corrosion, column destruction and blockage of the ESI capillary (Kaufmann et al., 2019). The use of ammonium fluoride is especially popular to enhance ionization of estradiol for mass spectrometric detection, showing benefits in both positive and negative modes (Fiers et al., 2012; Lindner, Vogeser, & Grimm, 2017). While the risks of pump wear and ionization suppression must be weighed against potential gains, many agree that ammonium fluoride in the μM range can impact estradiol sensitivity in a meaningful way

(Mezzullo et al., 2020). Furthermore, the inclusion of formic acid to an NH_4F-containing mobile phase is contraindicated, owing to the potential to generate hydrofluoric acid (Kaufmann et al., 2019).

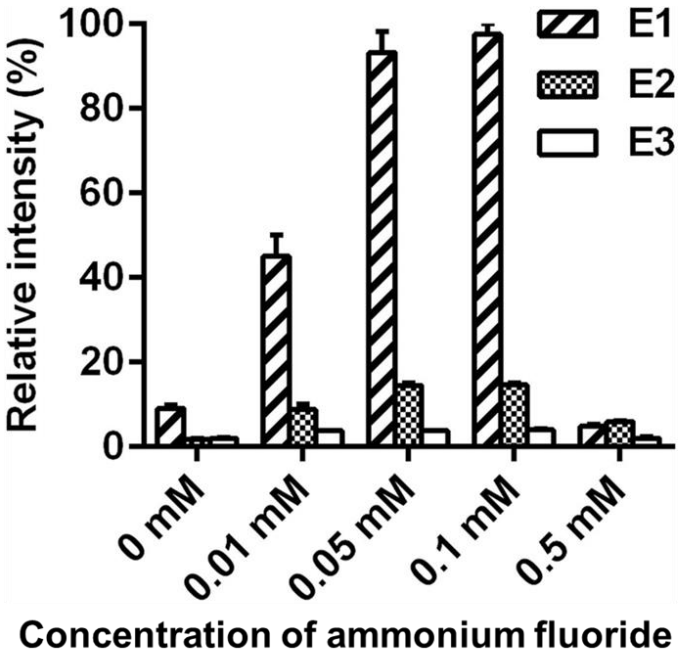

Figure 4.10: Signal enhancement of estrogens associated with ammonium fluoride mobile phase additive. Reprinted (figure 1) with permission from Li, X. S., Li, S., & Kellermann, G. (2018). Simultaneous determination of three estrogens in human saliva without derivatization or liquid–liquid extraction for routine testing via miniaturized solid phase extraction with LC–MS/MS detection. Talanta, 178, 464–472. https://doi.org/10.1016/j.talanta.2017.09.062

8. Mass Spectrometric Considerations

8.1. Testosterone

Positive electrospray ionization (+ESI) dominates in mass spectrum acquisition for testosterone, as shown in Table 4.1. However, positive-mode APCI (Gao et al., 2015; Gibson et al., 2019; Legacki et al., 2020; Xu et al., 2017) and APPI (Desai et al., 2019; Handelsman et al., 2020; Keski-Rahkonen et al., 2015) are also favored. The dominant ion transitions for the testosterone $[M + H]^+$ ion include m/z 289→109 and m/z 289→97. A representative +ESI mass spectrum for testosterone can be seen in Figure 4.11 (Zheng et al., 2019). In a comparison between ESI, APCI, and APPI, McCollough & Rob note a significant sensitivity gain (10×) with a field free-APPI (FF-APPI) source (McCulloch & Robb, 2017). FF-APPI is an orthogonal geometry APPI source, as opposed to an open geometry APPI source, which includes an extended reaction region (McCulloch, Robb, & Blades, 2016). In this study, APCI was most likely to enhance signal associated with plasma matrix (McCulloch & Robb, 2017). There were some differences in the ions produced among the three sources, with $[M - H_2O + H]^+$ seen at elevated temperatures in ESI and APCI. IN FF-APPI, an $[M - 2H_2O + H]^+$ ion, showing loss of two water molecules, was more prevalent, but not relevant to an assay that includes multiple hormones, as this m/z is identical to that of estradiol. (McCulloch & Robb, 2017). Selection of precursor ions for MRM is especially crucial in multianalyte steroid assays. Source temperature can be used to favor $[M + NH_4]^+$ or $[M + H - nH_2O]^+$ if $[M + H]^+$ does not produce a specific enough ion for identification (Gomez-Gomez et al., 2020).

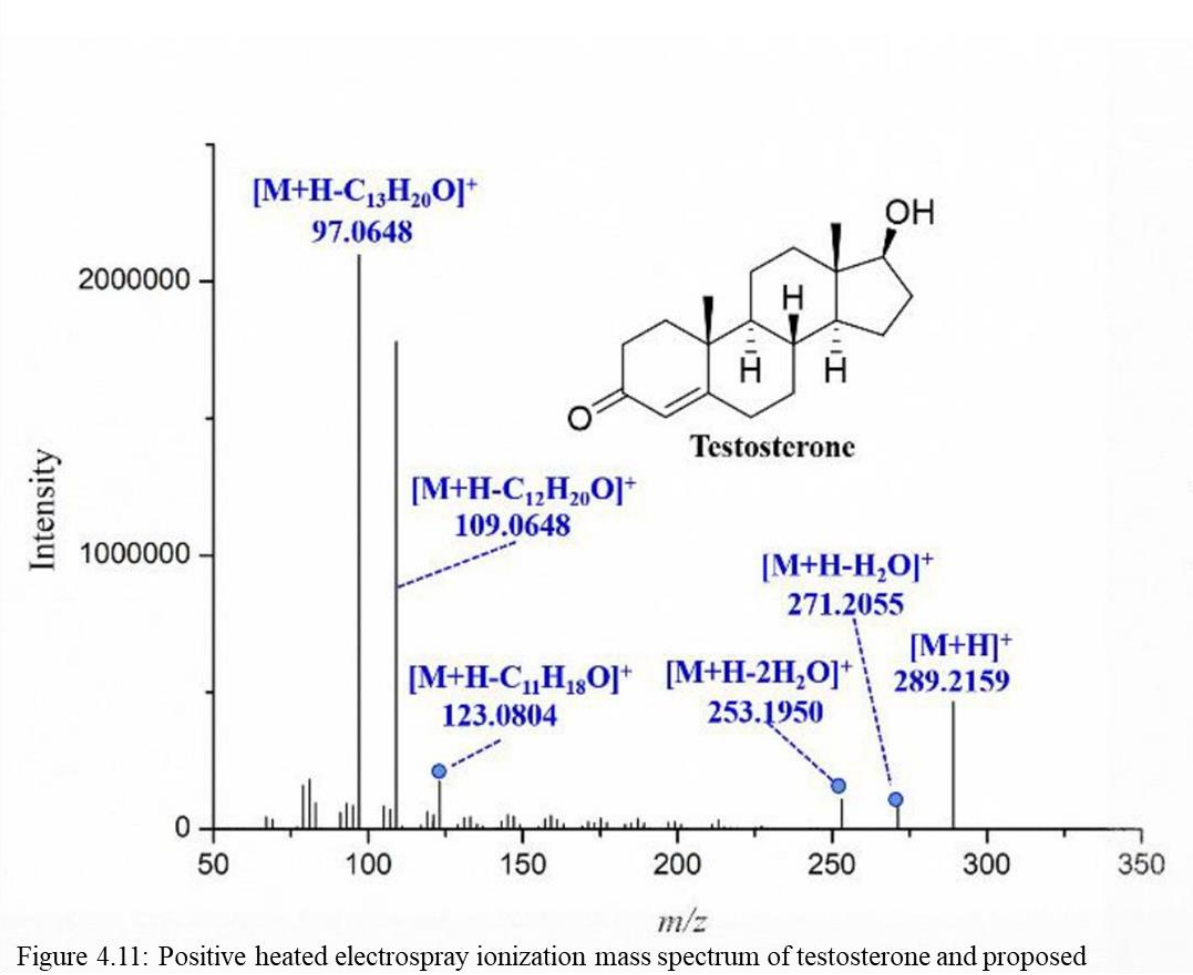

Figure 4.11: Positive heated electrospray ionization mass spectrum of testosterone and proposed fragmentation. Reprinted (portion of figure S3) with permission from Zheng, Y., Zhao, H., Zhu, L., & Cai, Z. (2019). Comprehensive identification of steroid hormones in human urine based on liquid chromatography–high resolution mass spectrometry. Analytica Chimica Acta, 1,089, 100–107. https://doi.org/10.1016/j.aca.2019.09.058

Several authors cite characteristic fragment ions and MRM transitions used to differentiate steroid hormones during analysis. Zheng and colleagues used steroids in urine as benchmarks for investigating diagnostic product ions (DPIs) for 13 steroid hormones that verify the validity of m/z 97.065, 109.065 and 123.080 for structures that lack C-11 substituents such as testosterone (Zheng et al., 2019). In contrast, MS/MS spectra from estrogens showed DPIs at m/z 107.049, 133.65 and 159.080. Of the steroids investigated in this study, all 13 formed at least one

dehydrated ion despite not all having a hydroxyl group (Zheng et al., 2019). Derivatization often affects steroid mass spectra. Production of oxime derivatives eliminated the appearance of sodium and potassium adducts seen in underivatized testosterone and progesterone (Keski-Rahkonen et al., 2011). This elimination of adducts helps increase the MS signal from the $[M + H]^+$ ion for the analytes, positively affecting sensitivity (Keski-Rahkonen et al., 2011).

8.2 Estradiol

As with testosterone, ESI, APCI and APPI sources can be utilized in estradiol quantification, but unlike testosterone, the MS source can operate in positive or negative ion mode depending on the use of derivatization in the sample preparation. Table 4.2 summarizes the m/z transitions unique to underivatized estradiol vs. the most common derivatization approaches. A mass spectrum for underivatized estrogens, collected in −ESI mode, is shown in Figure 4.13 (Zheng et al., 2019). Of the three most common ion sources, Keski-Rahkonen et al. explored which technique is most efficient for estradiol ionization using pure 17-β-estradiol buffer as well as using human serum (Keski-Rahkonen et al., 2013). They found that in all cases (−)APPI with a toluene dopant was the best ionization technique for 17-β- estradiol owing to the high sensitivity and resistance to background interferences inherent to biological matrices (Keski-Rahkonen et al., 2013). Other investigators have noted estradiol's poor ionization behavior in ESI and APCI (Khedr & Alahdal, 2016). This coupled with low water solubility makes mobile phase compatibility problem- atic, as well as mass spectrometric ionization (Ceglarek et al., 2009). Gibson and colleagues performed some extensive optimizations in ±ESI and ±APCI using selected ion monitoring (SIM) and parallel reaction monitoring (PRM) modes. There was noted higher signal intensity and peak area in APCI for estradiol and other steroids (Gibson et al., 2019). Similarly, APCI and FF-APPI outperformed ESI for matrix effects (McCulloch & Robb, 2017). Matrix effects can be

especially impactful owing to the low quantification limits that clinically useful estradiol LC–MS/MS demands. Matrix effect experiments showed ionization suppression for estradiol from endometriotic tissues, but showed enhanced signal in plasma, serum and endometrium homogenates (Häkkinen et al., 2019).

Table 4.2: MS/MS Transitions Published for Underivatized and Derivatized 17-β-Estradiol

Mass spec source	**Derivatization**	**MS/MS transition**	**Reference**
-ESI	None	271.1 è 144.9; 271.1 è 182.9	Mezzullo, 2020
+ESI/+APCI	None	273.3 è 107.0	Gibson, 2019
+ESI	Dansyl chloride	506.2 è 171.1	Ke, 2014
+ESI	Dansyl chloride	506.3 è 156.1	Ray, 2012
+ESI	2-FMP	364 è 128; 364 è 110	Faqehi, 2016
+APPI	DMIS	431 è 367; 431 è 96	Keski-Rahkonen, 2016
+ESI	NMPS	428.2 è 364.2	Wang, 2015
+ESI	PPZ/MPPZ	551.0 è 504.3; 551.0 è 58.3	Denver, 2019
+ESI	BMP	501.5 è 215.1	Khedr and Alahdal, 2016

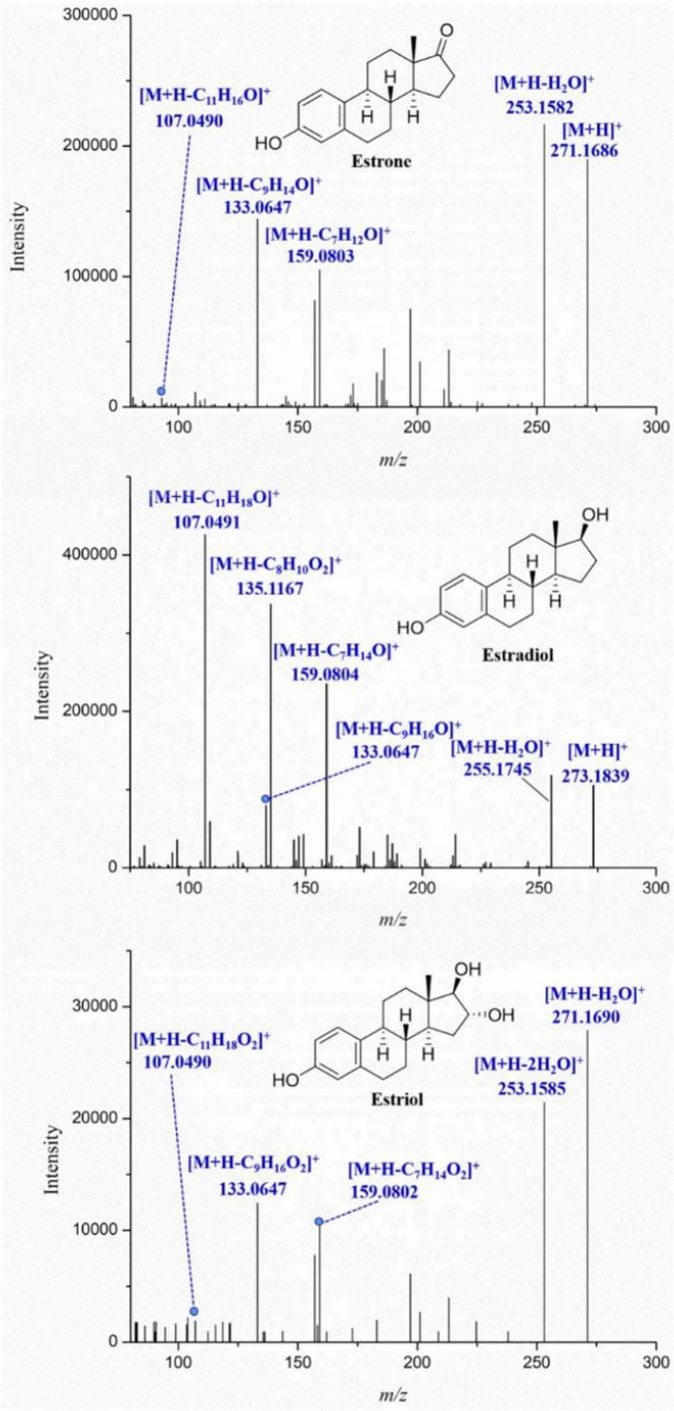

Figure 4.13: Negative heated electrospray ionization mass spectrum of underivatized estrogens and proposed fragmentation. Reprinted (figure S5) with permission from Zheng, Y., Zhao, H., Zhu, L., & Cai, Z. (2019). Comprehensive identification of steroid hormones in human urine based on liquid chromatography–high resolution mass spectrometry. Analytica Chimica Acta, 1,089, 100–107. https://doi.org/10.1016/j.aca.2019.09.058

The inclusion of differential ion mobility spectrometry (DMS) is uncommon in estradiol LC–MS/MS, but it could prove advantageous owing to its contribution to separating isobaric compounds and lowering background noise (Cohen et al., 2017). In an experiment comparing orthogonal separation of underivatized estrone, estradiol and estriol in eel tissue, investigators noted improved mass resolution when using DMS with −ESI vs. −ESI alone when given the inclusion of an isopropanol modifier (Cohen et al., 2017).

8.3 Progesterone

Unlike estradiol, derivatization is not often used to facilitate mass spectrometric detection for progesterone. Furthermore, derivatization reactions intended to boost ionization of other steroid analytes typically do not affect progesterone owing to the lack of suitable functional groups for the derivative (Galligan et al., 2018; Keski-Rahkonen et al., 2015; Yuan et al., 2019). Underivatized progesterone is detected well in positive ion mode, vs. negative mode, as verified by Zhang et al. (2008). The MRM transitions monitored are consistently in the area of m/z 315→109 and 315→97 in ESI (Gomez-Gomez et al., 2020; Schiffer et al., 2018), APCI (Gao et al., 2015) and APPI (Desai et al., 2019), but some investigators prefer the larger product ion m/z 297 (Hayden et al., 2017). The chemistry of these transitions is common to other steroid analytes, as most assays will monitor fragments associated with loss of water or breakage of the steroid back- bone (Surowiec et al., 2011). For progesterone, two successive losses of carbonyl oxygens contribute to the dehydrated ions m/z 297 and 279, as shown in Figure 4.12 (Zheng et al., 2019). Note that while not always affecting the structure of progesterone, some derivatization conditions can impact the formation of sodium and potassium adducts of progesterone, which can increase the presence of $[M + H]^+$ ions (Keski-Rahkonen et al., 2011).

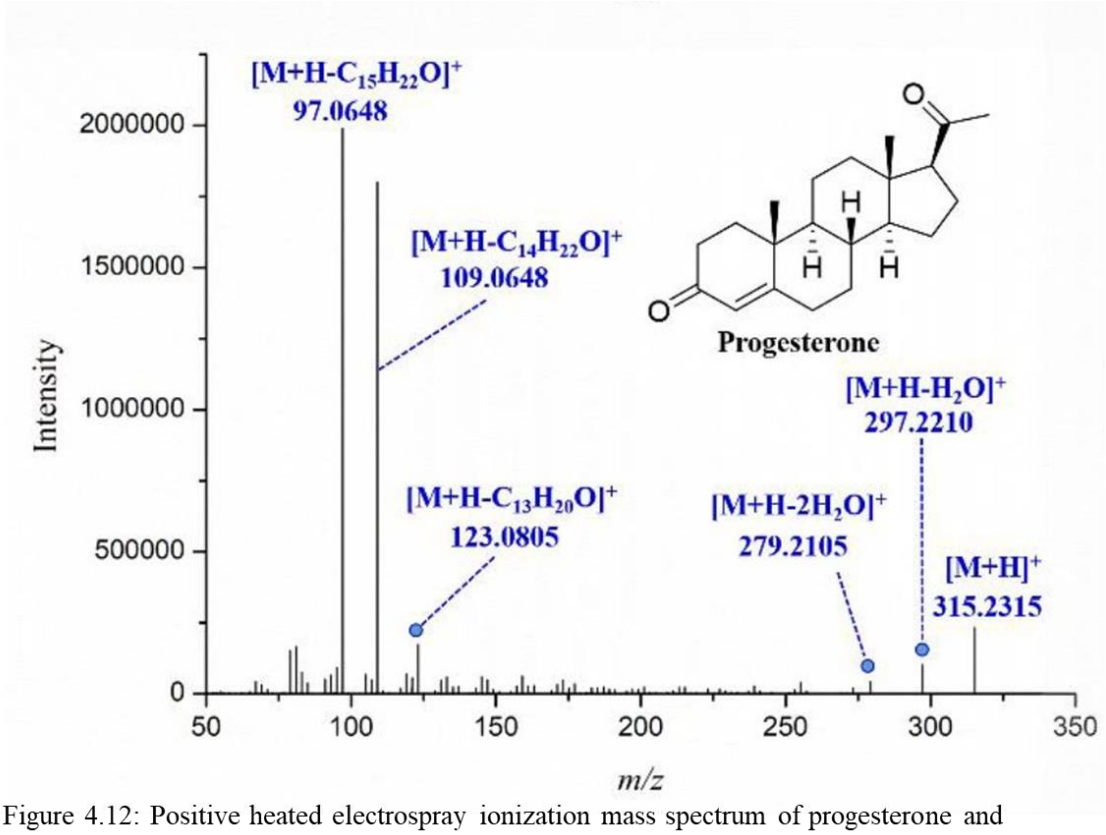

Figure 4.12: Positive heated electrospray ionization mass spectrum of progesterone and proposed fragmentation. Reprinted (portion of figure S3) with permission from Zheng et al. (2019). Comprehensive identification of steroid hormones in human urine based on liquid chromatography–high resolution mass spectrometry. Analytica Chimica Acta, 1,089, 100–107. https://doi.org/10.1016/j. aca.2019.09.058

9. Discussion

Testosterone, estradiol and progesterone are endogenous sex hormones that are critical for the development and maintenance of male and female sex characteristics. Measuring these hormones accurately and with high sensitivity is necessary to monitor the health of children, pre- and post-menopausal females, males and animals in veterinary care. Immunoassays have been used to measure endogenous hormones for decades; however, immunoassays can only measure one analyte at a time, and cannot compete with the accuracy and sensitivity of newer methods, most

notably, HPLC coupled with tandem mass spectrometry (LC–MS/MS). Mass spectrometry is increasingly being recognized as a “gold standard” tool for the measurement of testosterone, estradiol and progesterone in biological matrices in the endocrinology community (Conklin & Knezevic, 2020; Rosner et al., 2007). This technique allows for simultaneous quantification of testosterone, estradiol and progesterone, as well as other steroid hormones and metabolites. Human serum is the most widely used matrix for the LC–MS/MS quantification of endogenous sex hormones, followed by plasma. In fact, NIST methods for all three hormones utilize serum as the matrix (Tai et al., 2006; Tai et al., 2007; Tai & Welch, 2005). The most variable aspect of LC–MS/MS among testosterone, estradiol and progesterone is sample preparation. While testosterone and progesterone run well in positive ion mode following extraction (LLE, SPE), derivatization is often a key step for estradiol analysis. While estradiol can run in negative ion mode underivatized, higher sensitivity achieved in positive mode is predicated upon derivatization for this analyte (Denver et al., 2019). Furthermore, the need for high sensitivity is most relevant to estradiol, which can be found at clinically relevant low pg/ml levels in some patients. The lipophilicity of these hormones lends them to separation in reversed-phase mode, most often with C_{18} columns. Other phases, like biphenyl, are growing in popularity. Similarities in methodological parameters allow for the simultaneous quantification of many steroid hormones beyond the three discussed in this review. The most comprehensive method of steroid hormone quantification by LC–MS/MS included in this review achieved accurate measurement of more than 100 steroids simultaneously, including testosterone, estradiol and progesterone (Gaikwad, 2013). Several investigators quantified estradiol, testosterone and progesterone in serum, in addition to up to 17 other analytes (Desai et al., 2019; Genangeli et al., 2017; Lee et al., 2016; Qin et al., 2020). Similarly, Häkkinen et al. quantified over 20 analytes simultaneously

from endometrial tissue (Häkkinen et al., 2018). Urine was also a good matrix to capture simultaneous quantification of estradiol, testosterone and progesterone, plus many additional analytes (Kaabia et al., 2018; Son et al., 2020; Zhou & Cai, 2020). The examples of simultaneous steroid hormone measurement mentioned above, as well as the examples of ultra-sensitive single steroid hormone quantification discussed in the main text, contribute to the field's understanding of best practices for the quantification of testosterone, estradiol and progesterone. LC–MS/MS will probably be the standard for hormone quantification for the foreseeable future, although researchers will continue to push the limits of accuracy and sensitivity to maximize clinical relevance.

10. Conclusions

The importance of sensitivity and selectivity for hormone bioanalysis cannot be overstated, especially as our understanding of the roles estradiol, testosterone and progesterone play in normal development and disease manifestation evolves. One aspect of future development in this field will probably be the growth of multicomponent assays that include not only the hormones that are the focus of this review, but also their metabolites and synthetic precursors. Such assays produce highly complex datasets, suited to the fields of metabolomics and steroidomics, which are developing at a rapid pace. Moving forward, the field of steroid bioanalysis is also likely to see an increased respect for standardization for routine clinical analysis. The issue of clinical analysis of hormones was significant enough to attract the attention of the CDC, who since 2007 have been working toward more standardization in hormone quantification through the HoSt program. Many of the assays cited in this review springboard from the CDC work in this area, utilizing reference materials and methodology developed and validated by CDC scientists. For both future directions, continued improvements

in sensitivity needed to envelop all patient groups will hinge on advancements in and optimum utilization of LC–MS sample preparation, chromatography and instrumentation.

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CHAPTER 5. CONCLUSIONS

Female sex hormones demand attention when studying pathogens of the female genital tract, not only for their endogenous presence but because of the overwhelming use of hormonal contraceptives and hormone replacement therapy. Estrogen and progesterone influence host anatomy and physiology as well as the pathogenesis of infectious agents. Due to the short, 4-5-day estrus cycle of mice, *Chlamydia*-infected cells are typically shed from the genital tract before infection begins to ascend the genital tract following a physiologically relevant vaginal inoculation. Thus, hormone treatment to halt the estrus cycle has been used so that infection can be established and subsequently studied. *In vivo Chlamydia* genital infection has been studied under the influence of exogenous progesterone in the form of depot medroxyprogesterone acetate (DMPA) since at least 1981 (Tuffrey and Taylor-Robinson 1981). While these studies have pushed the field of *Chlamydia* research forward, DMPA has been shown to influence host immunity as well as pathogen biology (Tasker et al. 2020). This calls for an alternative to the basic model of *in vivo* genital *Chlamydia* research that allows for the study of *Chlamydia* without the effects of exogenous hormone treatment.

We began to characterize a mouse model that did not require DMPA treatment by delivering physiologically relevant levels of E2 and P4 to ovariectomized mice. The silastic capsule method of long-term E2 treatment of mice described by Ingberg, *et al.* in 2012 (Ingberg et al. 2012) was followed for E2 administration, and the same method was adjusted for the administration of P4 and combination of E2/P4. Using this model, we were able to study *C. muridarum* EB shedding trends and the presence of immune mediators in mice without DMPA pretreatment. Previous *in vitro* data from our lab showed that *C. trachomatis* infection was enhanced by E2 treatment and that the addition of P4 antagonized the expansion seen with E2-

only exposure in a human endometrial/stromal cell co-culture model (Kintner et al. 2015). Based on this report, we expected that an E2-dominant environment would enhance *C. muridarum* infection *in vivo*. Surprisingly, we found that E2 treatment, alone or in combination with P4, completely protected mice from *C. muridarum.* Our findings agreed with a similar study that found that E2 treatment protected rats from *C. muridarum* infection (Kaushic et al. 2000). We observed changes in the T cell response between E2-, P4-, and E2/P4-treated mice on day 10 post infection, but the lack of T cells measured from the E2- and E2/P4-treated mice is likely explained by the failure of *Chlamydia* infection to be established in these mice.

Estrogen primarily signals through two estrogen receptors (ER), ERα and ERβ. One component of the ER complex is protein disulfide isomerase (PDI). PDI is involved in activation of ERs (Landel et al. 1995), and *C. trachomatis* EB are associated with host PDI (Davis et al. 2002). These *in vitro* findings suggest that ERs are involved in chlamydial host cell entry through PDI-EB interactions. We used ERα or ERβ KO mice to determine if the absence of ERs *in vivo* would reduce *Chlamydia* shedding or affect host pathology or immune response. We saw that the absence of ERα in mice shifted the timing of EB shedding compared to ERβKO and WT mice, indicating that ERα signaling is involved in chlamydial infection. To begin to determine how ERα is involved in chlamydial infection, we examined immune cell and cytokine presence in the genital tracts. Using flow cytometry, we found that at day 9 post infection (pi), ERαKO mice infected with *C. muridarum* had higher numbers of T cells. Additionally, using targeted RNAseq, we found that ERαKO mice had upregulated CD4 and Foxp3. Together, these data showed that ERαKO mice had increased regulatory T cell presence at day 9pi compared to ERβKO and WT mice. Since we saw that the ERαKO mice shed EB more quickly than the ERβKO and WT mice, the regulatory T cells could have infiltrated more quickly as well. A

previous study found that increased regulatory T cells during *C. muridarum* infection in mice could promote the production of Th17 cells to aid in the clearance of *C. muridarum* (Polanczyk et al. 2004). While our studies reveal the importance of regulatory T cells in the clearance of chlamydial infection in mice, further studies examining the T cell presence at additional timepoints post infection are required to make more definitive conclusions on how ER signaling influences the immune response to *C. muridarum* infection.

Due to the increase in genital *Chlamydia* cases reported each year and the massive number of women using hormonal contraceptives or hormone replacement therapy, the study of female sex hormone-*Chlamydia* interactions must continue. The studies described in chapter 3 should be continued to determine immune responses under different hormonal conditions at several more time points after chlamydial infection. Those studies were limited to one time point post infection (day 9 or 10), providing a snapshot of the total immune response to *C. muridarum* infection in the mouse genital tract. Repeating the flow cytometry and RNAseq studies to determine the presence of immune mediators earlier (days 3 and 6) and later (days 12 and 15) post infection will allow for more extensive comparisons of what is happening under different hormonal conditions at different times. Additionally, our ER knockout studies were limited to observing the effects of the absence of either ERα or ERβ alone. Future studies should use an ERαKO mouse treated with ICI 182,680, an ER antagonist that has been shown to silence ERβ function (Hou et al. 2004). This would allow for the complete silencing of ERα and ERβ signaling to ensure that one receptor is not making up for the lost signaling of another in the single knockout models.

The data we presented in chapter 3 highlight the contributions of hormone stimulation on the immune response, but our previous *in vitro* work showed that epithelial and stromal cells are

also influenced by E2 and P4 (Hall et al. 2011; Kintner et al. 2015). Together, these studies indicate that the hormonal influence of *Chlamydia* infection is multifactorial, so future studies using ERKO or ovariectomized mice should be used to examine the roles of epithelial and stromal cells on *C. muridarum* infection *in vivo.*

The *Chlamydia* research community must address the issue of differential hormonal interactions in mice versus humans and consider these differences when studying *Chlamydia* using the murine model. We used silastic capsules to deliver hormones to ovariectomized mice in order to bypass the need for DMPA treatment. However, there are other animal models to consider. The common spiny mouse (*Acomys cahirinus*) was described in 2016 as having a human-like menstrual cycle, the only known rodent of its kind. The spiny mouse has an average estrous cycle of 8.7 days with the typical murine stages (proestus, oestrus, metestrus, and diestrus), and an additional 3 day stage characterized by large numbers of red blood cells, a phase not typically seen in rodents (Bellofiore et al. 2017). The spiny mouse has natural the natural remodeling of endometrial tissues seen in mammals accompanied by spontaneous decidualization (Bellofiore et al. 2018). This model is currently being used for the study of abnormal menstruation and pregnancy (McKenna et al. 2020; Mckenna et al. 2021), but could be a good candidate for the study of *Chlamydia* without exogenous hormonal treatment. An established alternative *Chlamydia* model is the guinea pig. *C. caviae* is a chlamydial species that causes guinea pig inclusion conjunctivitis (GPIC). Studies have shown that E2 treatment of guinea pigs enhanced infection by making it last longer and have more EB shedding, which agrees with previous *in vitro* studies that showed E2 enhancement of *C. trachomatis* infection of human epithelial cells (Kintner et al. 2015). Guinea pig reproductive biology is more similar to humans; they have a 15-17 day estrus cycle and a 59-72 day gestation period (Suckow et al.

2012; Shomer et al. 2015 Jan 1). Historically, guinea pig models have been hampered due to a lack of reagents. However, the development of new techniques and commercially available reagents make this model worth revisiting when studying the effects of hormones on chlamydial infection.

Lastly, the studies of female sex hormone interactions with *Chlamydia* should not be limited to natural estrogens and progesterone. Hormonal contraceptives and hormone replacement therapies utilize synthetic estrogens and progestins, and these should be studied with respect to chlamydial infection due to the rising number of individuals using these treatments. Synthetic hormones can be delivered to the body in different doses and could have different, off-target effects on the host and on *Chlamydia* compared to natural hormones. For example, as discussed earlier, DMPA, a synthetic progesterone, can influence immune action. Ethinyl estradiol is a common synthetic estrogen and norethindrone acetate, medroxyprogesterone acetate, and drospirenone and common synthetic progestins that should be considered when studying the influence of female sex hormones on *Chlamydia* infection.

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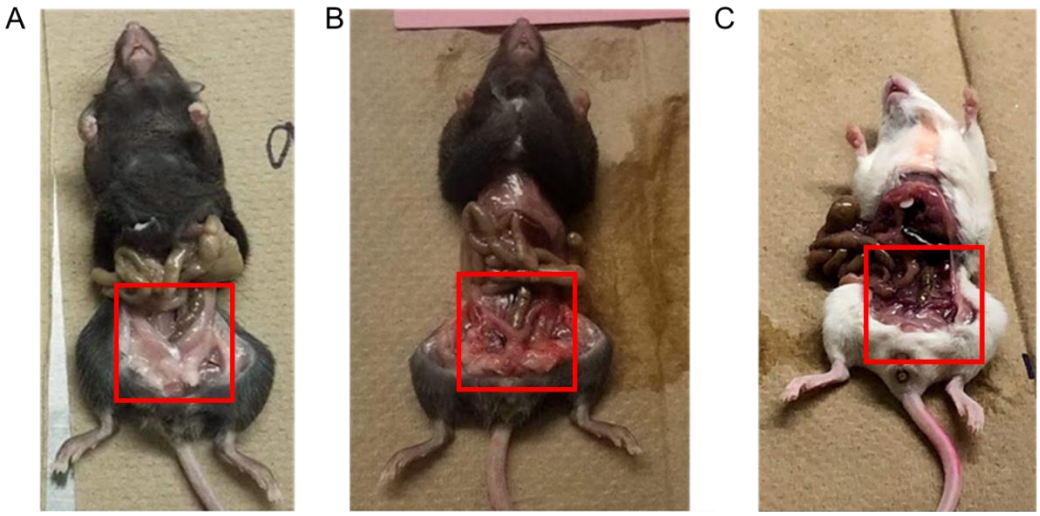
APPENDICES

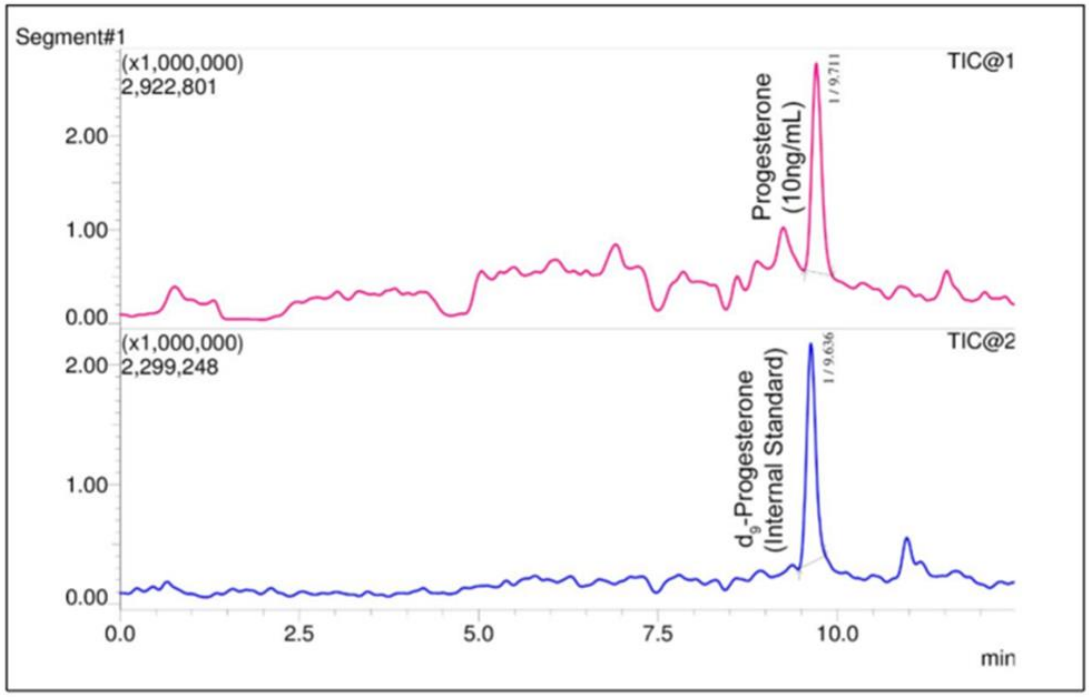
APPENDIX A: Chapter 3 Supplemental Figures

Supplemental Figure 3.1:



Supplemental Figure 3.1: Representative photos of genital tract scoring. These photos represent scores of **A.** 1, **B.** 3, and **C.** 4.

Supplemental Figure 3.2:



Supplemental Figure 3.2: Mass chromatogram of progesterone (315.01 m/z) and d_9-progesterone (324.12 m/z).

APPENDIX B: Summary of Analytical Parameters for Surveyed LC-MS/MS Methods Used in the Quantification of Testosterone, Progesterone, and 17-β Estradiol from Biological Matrices

Table 4.1. Summary of analytical parameters for surveyed LC-MS/MS methods used in the quantification of testosterone, progesterone, and 17-β estradiol from biological matrices

Citation	Analyte(s)	Matrix	Sample Preparation	LC Column	Mobile Phase	MS	Range	Application Notes
Beinhauer, 2015	E1, E2s (α/β), E3	Fetal bovine serum	Derivatization with 2-FMP	Kinetex XB-C18 (2.1x 100mm, 2.6micron)	Gradient (A: H_2O with 2.5% v/v FA; B: MeOH, 20 mM AF)	+ESI with MRM	5 - 1000 pg/mL	Stable-isotope internal standards; WCX RAM Trap column used to remove proteins prior to injection ("trap-and-elute")
Bichon, 2012	E2-17-esters	Bovine hair	Sonication and extraction followed by derivatization with DC	Acquity BEH C18 and Acquity BEH Phenyl (2.1x100mm, 1.7micron)	Gradient (A: H_2O with 0.1% FA; B: ACN with 0.1% FA)	+ESI with MRM	2.5 - 200 ng/g	Norethindrone acetate, ethinyl E2, and stanolone benzoate used as internal standards; method potentially useful for monitoring 'natural' hormone doping in livestock

Bose, 2013	E1, T, dexa meth-asone	Human plasma (200µL)	PP with ACN followed by drying and reconstituting in mobile phase	Shodex C18 (4.6x100mm, 3 micron)	Isocratic (A: 0.1% FA in H_2O; B: ACN)	Positive (+) dual ESI	2 - 12 µg/mL	Compared method validation between HPLC assays referenced in pharmacopeias and LCMS
Bothello, 2013	T	Human serum (500µl - 1mL)	PP with AA and LLE with hexane-ethyl acetate (2:3)	C18 Hypersil Gold (3.0x 50mm, 3micron)	Gradient (A: H_2O; B: ACN, each with 0.1% FA)	+ESI with MRM	0.35 - 5 ng/mL	Stable-isotope internal standards; candidate RPM for T as part of CDC HoST program
Broccardo, 2013	T, DHP, P, cortisol, cortisone	Human serum (100µL)	PP with MeOH in 96-well plate	Trizaic nanotile (150µMx 50mm) with BEH C18 packing	Gradient (A: H_2O with 0.1% FA; B: MeOH with 0.1% FA)	+ESI with MRM	1 - 250 mg/mL	Stable-isotope internal standards; Compared microfluidic LC-MS/MS assay to antibody-based measurement system; Significant reduction in solvent consumption and sample size versus

								traditional LC-MS/MS
Cegearak, 2009	Total of 9 steroids, including T, P, and 17β-E2	Human serum (100μL)	On-line SPE (Poros R1)	Chromo-lith RP-18 (4.6x 250mm)	Gradient (A: H_2O; B: MeOH)	+/- ESI, APCI, and APPI with MRM	25 - 7000 ng/mL	Stable-isotope internal standards; compared 3 mass spec sources and ultimately chose APCI, compared results to immunoassay
Cohen, 2017	E1, 17β–E2, E3	Eel tissue	Homogeni-zation with 150mM sodium acetate buffer, PP with ACN, hexane	Luna C18 (3x150mm, 100A)	Gradient (A: H_2O with 0.05% NH4OH; B: ACN) 10 - 200 ng/mL	DMS with IPA; -ESI with SRM	1 - 1x106 pg/mL	Applied to study of eels treated with E2 to meet regulatory compliance of hormone residue; s/n ratio significantly improved with use of DMS versus -ESI alone
Denver, 2019	Total of 9 estrogens including E1 and α/β–E2	Human plasma (0.5mL)	SPE (Oasis MCX) followed by derivatization with PPZ and quaternizat	ACE 2 Excel C18-PFP (2.1x150mm, 2 micron)	Gradient (A: H_2O; B: ACN each with 0.1% FA)	+ESI with MRM	1 - 2000 pg/mL	Stable-isotope internal standards; assay applied to compare

			ion by methylation (MPPZ)					control versus PAH-exposed females to capture snapshot of estrogen metabolism
Desai, 2019	E1, 17β-E2, P, T, plus 14 others	Mouse serum (200μL)	LLE with MTBE	Restek Raptor biphenyl (2.1x100mm, 2.7micron)	Gradient (A: H_2O; B: MeOH; C/dopant: toluene)	+/- APPI with MRM	0.02 - 16 ng/mL	Stable-isotope internal standards; first method to report quantification of estrogens and androgens in both "classical" and "backdoor" biosynthetic pathways (18 total hormones)
Faqehi, 2016	E1, 17β-E2	Human plasma/ serum (0.5 - 2mL)	SPE (Oasis MCX) followed by derivatization with 2-FMP	Acquity BEH C18 (2.1x 50mm, 1.7micron)	Gradient (A: H_2O; B: 0.1% FA in MeOH)	FT-ICR MS; +ESI with MRM	2 - 800 pg/mL	Stable-isotope internal standards; improved specificity for estrogens demonstrated versus DC

								derivatization
Farke, 2011	Androgens (including T), P, free/ conjugated estrogens (including 17β-E2)	Bovine colostrum and colostrum products	Hydrolysis (β-glucuronidase), LLE with MTBE/petroleum ether (30:70, v/v)	Luna C18 (3x150mm, 3 micron) Acquity BEH Shield RP18 (2.1x100mm, 1.7 micron)	Gradient (A: H_2O + additive; B: MeOH + additive)	+/- ESI	0.1 - 1 ng/mL	Assay demonstrated that hormone concentrations in colostrum were higher than other commercial food products; used different mobile phase additives for androgens and P versus estrogens
Fiers, 2012	17β-E2 and E1	Human serum (500µl)	LLE with hexane-ethyl acetate (9:1)	Supelguard LC-8-DB (3x20mm) and Supelco LC-8-DB (2.1x330mm, 3micron)	Gradient (A: H_2O with 0.2mM NH_4F; B: MeOH)	-ESI with MRM	0 - 2000 pg/mL	Stable-isotope internal standards; 2D LC configuration improved sensitivity for underivatized E2 (< 0.5 pg/mL)

Gaikwad, 2013	101 steroids and estrogen-related compounds, including 17 β-E2, P, T	Breast tissue (100mg)	PP with MeOH	Acquity HSS T3 (1.0x 150mm, 1.8 micron)	Gradient (A: H_2O; B: ACN each with 0.1% FA)	+ESI with MRM	0.05 - 1000 ng/mL	Assay provides a comprehensive steroid and steroid metabolite profile with 12 min run time
Galligan, 2018	E1, 17β-E2, P, T, plus 7 others	Dolphin plasma	PP with MeOH, SPE with Supelclean LC-18 followed by derivatization with DC	Restek Ultra biphenyl (4.6x250mm, 5 micron)	Gradient (A: 0.1% FA in MeOH; B: 0.1% FA in ACN)	+ESI with MRM	0 - 25 ng/g	Stable-isotope internal standards; assay applied to investigation of circulating hormone levels un free-roaming bottlenose dolphins
Gao, 2015	17β-E2, P, T, plus 4 others	Human saliva (100μL) collected using Salivette device	PP with in-line SPE (Chromolith Speed ROD RP-18e)	Shim-pack XR ODS (3x75mm, 2.2 micron)	Gradient (A/B: MeOH/ H_2O with 2.0M AA)	+APCI	0.001 - 10 ng/mL	Stable-isotope internal standards; assay offers a novel high throughput (5.2 min/sample) quantification of hormones in saliva

Genangeli, 2017	E1, 17β-E2, P, T, plus 14 others	Equine serum (400μL)	PP with ACN	Zorbax RRHD C18 (2.1x50mm, 1.8 micron)	Gradient (A: 0.1% FA in H_2O; B: 0.1% FA in ACN)	+/- ESI with MRM	0.002 - 5.5 ng/mL	Stable-isotope internal standards; assay is fully validated, including an evaluation of matrix effects using post-column infusion
Gibson, 2019	E1, 17β–E2, E3, T, P, plus 9 others (some metabolites)	Human tears (100μL)	3 compared: PP with cold ethanol, LLE with MTBE/diethyl ether, and Schirmer strips	Acquity BEH C18 (1.0x 150mm, 1.7micron)	Gradient (A: H_2O; B: ACN, both with 10mM AF)	+/- APCI with SIM and PRM	0.1 - 1000 ng/mL	Extensive method optimization (ESI versus APCI; extraction) provides novel method for hormone quantification in tears; limitation in need to pool samples
Gomez-Gomez, 2020	P, T, plus 23 others (some metabolites)	Amniotic fluid (50μL), saliva (1mL), breast milk (1mL)	PP (amniotic fluid) followed by LLE with ethyl acetate; saliva and breast milk LLE	Acquity BEH C18 (2.1x 100mm, 1.7 micron)	Gradient (A: H_2O; B: ACN, both with 1mM AF and 0.01% FA)	+ESI with SRM	0.02 - 3000 ng/mL	6 difference stable-isotope internal standards used to cover 25 analytes; authors

			with ethyl acetate					suggest method would be useful for establishing endogenous levels of steroids in unique matrices
Häkkinen, 2018	Androgens (including T), progestins (including P), estrogens (including 17β-E2), and corticosteroids	Serum, plasma, endometrium tissue homogenate (150μL)	LLE with toluene	Kinetex Biphenyl (2.1x100mm, 1.7 micron)	Gradient (A: H_2O; B: MeOH/H_2O 95:5, both including 0.2mM NH_4F)	+/- ESI with MRM	0.01 - 1000 nM	Stable-isotope internal standards, of the 27 steroids included in method development, 11 suitable for quantification; sensitivity appropriate for premenopausal women
Handelsman, 2020	E1, 17β-E2, T, DHT	Serum (200μL)	LLE with MTBE	Kinetex XB C18 (2.1x 50mm, 1.7 micron)	Gradient (A: H_2O; B: MeOH)	+/- APPI with MRM	0 - 100 ng/mL	Stable-isotope internal standards; used assay to compare serum samples stored for 10 years, 17β-E2 most

								affected by storage and freeze/thaw
Hauser, 2008	E1, pregnane-diol, cortisol, T and metabolites	Primate urine (200µL)	β-glucuronidase treatment followed by LLE with MTBE	Gemini C18 (2x150mm, 3 micron)	Gradient (A: H_2O/ACN 95/5; B: H_2O/ACN 5/95)	+/- ESI with MRM	0.1 - 1000 ng/mL	Stable-isotope internal standards; relevant endogenous levels of steroids in primates
Hayden, 2017	P, T, hydro-cortisone	Whale blubber (50 mg)	PP with ACN and LLE with hexane	Pepmap RSCL (15x75µm, 2 micron)	Gradient (A: H_2O; B: ACN both with 0.1% FA)	nano ESI with SRM	0.05 - 10 nM	Cholic acid used as internal standard; assay allows for high sensitivity quantification of steroids from whale biopsy samples
Higashi, 2011	P	Human saliva (400µL)	PP with ACN and SPE with Strata-X	J'sphere ODH-8 (2x150mm, 4 micron)	Isocratic (A: 10mM AF; B: MeOH)	+ESI with SRM	12.5 - 250 pg/mL	Deuterium-labeled P internal standard; assay used to study P saliva fluctuations in menstrual cycle and

								pregnancy
Higashi, 2017	T, cortisol, plus 3 others	Fingernail clippings (10mg)	Incubation (ethanol/H2O) with ultrasonic extraction and SPE with Strata-X or Oasis HLB	J'sphere ODH-8 (2x150mm, 4 micron)	Gradient (A: 20mM AF; B: MeOH)	+/- ESI with SRM	1 - 100 ng/mL	Stable-isotope internal standards; assay revealed handed differences in hydrophilic steroids, sex differences with T concentration in fingernails
Hogg, 2005	T	Bottlenose dolphin saliva and blow	Sample preserved with magnesium chloride and SPE with Envi-Chem P cartridges	Alltech Macrosphere C8 (2x150mm, 5 micron)	Isocratic (A: H_2O with 0.5% CH_3COOH; B: 90% ACN with 0.5% CH_3COOH)	+ESI with SIM	0.5 - 50 ng/mL	Surrogate internal standard; assay more sensitive than RIA techniques and not affected by cotton-based sampling systems
Kaabia, 2018	Total of 44 conjugated and free steroids, including T, P, and 17β-E2	Bovine urine	SPE with Isolute C18 and Oasis HLB	Acquity CHS C18 (1.0x 150mm, 1.7 micron)	Gradient (A: H_2O; B: ACN both with 0.1% CH_3COOH)	+/- HESI with XCMS post-processing	0.01 - 10 ng/mL	Assay part of development of a steroidomic work flow; applied to the

								analysis of bovine urine serum following boldenone administration
Kaufmann, 2019	E1, 17β–E2, E3, T, plus 38 others	Bovine urine, muscle, serum, whole blood (5g)	β-glucuronidase treatment followed by LLE with ethyl acetate and hexane with pH adjustment	Kinetex C18 (2.1x150mm, 2.6 micron)	Gradient (A: H_2O/ACN 95/5; B: H_2O/ACN 5/95 both with 2% 100mM NH_4F)	+/- ESI with full-scan and target-ed MS/MS	0.25 - 10 μg/kg	Matrix-matched external calibration; unique features of sample preparations (pH adjustment) and chromatography investigation (NH_4F)
Ke, 2014	E1, 17β-E2, T, DHT, plus 4 others	Human serum (post-menopausal; 500 μL)	LLE with 1-chlorobutane followed by derivatization with DC	Zorbax SB-C18 Rapid Resolution HD (2.1x50 mm, 1.8 micron)	Isocratic (A: H_2O with 0.2% FA; B: MeOH)	+ESI with MRM	1 - 2500 pg/mL	Stable-isotope internal standards; derivatization specific for estrogens; validated assay applicable to clinical use
Keski-Rahkonen, 2015	17β-E2, T, DHT	Human serum (200μL)	LLE with MTBE followed by derivatization with DMIS	Kinetex Phenyl-Hexyl (2.1x100mm, 1.7 micron)	Gradient (A: H_2O; B: MeOH)	+APPI with MRM	0.01 - 16 ng/mL	Stable-isotope internal standards; 120 patient serum

								samples analyzed via current and previous assay with 10x increase in E2 sensitivity with DMIS derivatization
Keski-Rahkonen, 2011	P, T, plus 5 others	Human serum (150μL)	LLE with MTBE followed by derivatization with hydroxylamine	Zorbax SB-C18 (2.1x 50mm, 1.8 micron)	Gradient (A: H_2O; B: MeOH)	+ESI with MRM	0.033 - 80nM	Mass spectral selectivity improved for T and 2 others using hydroxylamine derivatization; simple sample preparation workflow
Keski-Rahkonen, 2013	17β-E2	Human serum, endometrial tissue homogenate (150μL)	LLE with MTBE	Poroshell 120 SB-C18 (2.1x50mm, 2.7 micron)	Gradient (A: H_2O; B: MeOH)	+/- APCI, APPI, and ESI	0.025 - 50nM	Stable isotope internal standard; negative (-) APPI with toluene dopant produced highest sensitivity for 17-E2

Khedr and Alahdal, 2016	E1, 17β−E2, E3, plus 7 others (some metabolites)	Human serum from healthy and non-invasive breast cancer women (250μL)	SPE with Chromobond C8 followed by derivatization with BMP	Zorbax Extend C18 (4.6x150mm, 4 micron)	Gradient (A: H_2O with 0.1% FA; B: ACN)	+ESI with MRM	0 - 600 pg/mL	BMP derivatization enabled femtogram level sensitivity for estrogens compared to dansylation (DC)
Kushnir, 2009	E1, E2s (α/β), E3, T, plus 10 others	Ovarian follicular fluid (40μL)	LLE with MTBE followed by derivatization with DC or hydroxylamine	Varied (3 different columns used)	Varied based on column	Positive (+) TurboIon-Spray with MRM	Not stated; some < 1ng/mL	Stable-isotope internal standards; Combination of assays used to evaluate steroid concentrations in serum samples of 202 patients with cardiovascular disease (Naessen, 2010)
Laforest, 2019	E1, 17β−E2, cortisol, cortisone	Adipose tissue (200mg)	SPE with C18 Sep-Pak followed by derivatization with MPPZ and methyl iodide	ACE 2 Excel C18-PFP (2.1x150mm, 2 micron)	Gradient (A: H_2O; B: ACN both with 0.1% FA)	+ESI with MRM	5 - 1000 pg/ sample	Novel MPPZ derivative was specific to phenolic group on estrogens, applied to study of adipose tissue in

								health versus breast cancer positive women
Lee, 2016	Total of 12 free and sulfated steroids, including T, P, and 17β-E2	Human serum (100μL)	SPE with Strata-X	Unison UK phenyl C18 (2x50mm, 3 micron)	Gradient (A: H_2O; B: ACN each with 0.1% FA)	+/- ESI with SIM and MRM	0.1 - 50 ng/mL	Stable-isotope internal standards; assay applied to the study of steroid profiles in obese girls
Legacki, 2019	E1, 17β-E2, DHEAS	Equine plasma (500μ*L)*	PP with ACN/CH_3COOH	Eclipse XDB-Phenyl (2.1x50mm, 5micron)	Gradient (A: H_2O with 5mM NH_4F; B: MeOH)	-ESI with MRM	1 - 2500 ng/mL	Stable-isotope internal standards, applied to study of hormone concentrations during pregnancy in horse
Legacki, 2020	P, T, plus 3 others (some metabolites)	Serum from captive killer whales, bottlenose dolphins, belugas	LLE with MTBE	Acuity C18 (2.1x50mm, 1.8 micron)	Gradient (A: H_2O with 0.2% FA; B: MeOH)	+APCI with MRM	0.1 – 100 ng/mL	Stable-isotope internal standards, analytes monitored in early, mid-, and late pregnancy to investigate diversity of steroid metabolis

								m in toothed cetaceans
Li, 2015	E1, 17β–E2, E3, plus 15 others (some metabolites)	Human serum (100µL)	LLE with MTBE followed by derivatization	Ascentis Express C18 (3x150mm, 2.7 micron)	Gradient (A: H_2O; B: ACN both with 0.1% FA)	+ESI with targeted SIM	0.1 – 3000 pg/mL	Stable-isotope internal standards; compared four derivatization schemes to achieve sub 1pg/mL sensitivities
Li, 2018	17β-E2, E1, E3	Human saliva (250µL)	SPE with Oasis HLB	H2Os BEH C18 XP (2.1x100mm, 2.5 micron)	Gradient (A: H_2O with 0.1mM NH_4F; B: ACN)	-ESI with MRM	1 - 1000 pg/mL	Stable-isotope internal standards for all analytes; method underwent extensive optimization, include SPE conditions and NH_4F concentration
Matysik and Liebisch, 2017	P, T, plus 6 others	Human serum (100µL)	LLE with MTBE	Kinetex Biphenyl (2.1x50mm, 2.6micron)	Gradient (A: H_2O / MeOH 95/5; B: MeOH both with 0.1% FA)	+ESI with PRM	Not stated; LOQs < 1ng/mL	Stable-isotope internal standards; short run time (5.3min) with high resolution separation

								and detection using Orbitrap
McCulloch and Robb, 2017	Total of 7 steroids, including T, P, and 17β-E2	Human serum, plasma, & simulated urine (600μL)	PP with MeOH	Luna C18 (2.0x100mm, 3micron)	Isocratic (A: H_2O with 0.05% FA; B: MeOH, 10/90)	ESI, APCI, & field free APPI (FF-APPI)	0.1, 1ppm used for optimi-zation	Assay goal to compare MS sources; results indicate lower matrix effects in APCI and FF-APPI and at lower temperature/higher flow rates for nebulizing gas
Mezzullo, 2020	E1, 17β–E2, DHT, 17-hydroxy-pregnenolone	Human serum (300μL)	LLE with hexane: ethyl acetate (9:1) followed by in-line SPE	Gemini C6-Phenyl (2.0x 100mm, 3 micron)	Gradient (A: 20mM NH_4F in H_2O; B: MeOH)	+/- ESI with MRM	4.9 - 5000 pg/mL	Stable-isotope internal standards, LC-MS/MS compared to immunoassay to establish reference intervals
Nair, 2018	17β–E2 and NOMAC	Human plasma (500μL)	SPE with LiChroSep DVB-HL cartridges followed by derivatizati	Acquity BEH C18 (2.1x 50mm, 1.7micron)	Isocratic (A: 3mM amm. triacetate in H_2O; B: ACN)	+ESI with MRM	0.01 - 10 ng/mL	Stable-isotope internal standards; Assay used to determine PK profile of orally

			on with DC					administered steroids in postmenopausal women
Olisov, 2019	P, T, plus 6 others	Human serum (400μL)	LLE with MTBE	Unison UK C8 (2.1x 50mm, 3micron)	Gradient (A: H_2O; B: MeOH both with 0.2% FA)	+ESI with MRM	0.01 - 10 ng/mL	Stable-isotope internal standards; Assay applied to investigation of steroid levels in children before adrenarche, comparison between LC-MS/MS and immunoassay
Qin, 2020	Total of 29 free steroids, including T, P, and 17β-E2	Human serum (100μL)	Spilt sample; LLE with dichloromethane followed by derivatization with DC and butylamine or methoxyamine	Acquity BEH C18 (2.1x 100mm, 1.7micron)	Gradient (A: H_2O; B: ACN both with 0.1% FA)	+ESI with MRM	0.1 - 25 ng/mL	Stable-isotope internal standards; unique workflow involving a split sample, multiple derivatizing agents and re-combined sample for analysis

Ray, 2012	17β–E2	Human serum (250µL) and dialysis solution (200µL)	LLE with MTBE followed by derivatization with DC or hydroxylamine	Phenomenex C6 Phenyl (2.1x100mm, 3 micron)	Isocratic (A: 10mM FA in H_2O; B: MeOH)	+ESI with MRM	0.5 - 10 pg/mL	Stable-isotope labeled E2 internal standard; Assay applied to investigation of free E2 concentrations in women using equilibrium dialysis
Schiffer, 2018	P	Human saliva (300µL)	SLE+ with DCM elution and reconstituted in 40% MeOH	Kinetex C8 (3.0x100mm, 2.6 micron)	Gradient (A: H_2O; B: MeOH each with 0.1% FA and 2mM AA)	+ESI with MRM	0 - 10,000 pmol/L	Stable-isotope internal standard; Utilized for mapping P profile during menstrual cycle
Schofield, 2017	17β–E2, T	Human serum (500µL)	LLE with hexane: ethyl acetate (9:1)	Accucore C18	Gradient (A: 0.2 mM NH_4F in H_2O; B: ACN)	+/- ESI with MRM	25 - 750 nM	Stable-isotope labeled internal standards; cross-referenced validation using samples from CDC HoST program

Son, 2020	Total of 39 free and conjugated steroids, including T, P, and 17β-E2	Human urine (2mL)	β-glucuronidase treatment followed by SPE with Oasis HLB	Acquity BEH C18 (2.1x10mm, 1.7micron)	Gradient (A: H_2O; B: MeOH both with 0.01% FA and 1mM AF)	+ESI with MRM	0.3 - 8000 ng/mL	Stable-isotope internal standards (4); assay applied to analysis of urine samples from 5 male volunteers
Star-Weinstock, 2012	T	Human serum and dried blood spots (DBS)	LLE with diisopropyl ether followed by derivatization with QAO	Cadenza-CL (4.6x50mm, 3 micron)	Gradient (A: 0.1% FA in H_2O; B: ACN)	+ESI with MRM	1 - 5000 pg/mL	Stable-isotope internal standard; unique derivatization scheme for T showing significant signal enhancement versus underivatized
Surowiec, 2011	Total of 11 steroids, including T, P, and 17β-E2	Mouse tissue (adrenal gland, testicle, prostate, ovary)	Homogenization and PP with MeOH followed by SPE with Oasis HLB	Hypersil Gold (2.0x50mm, 1.9 micron)	Gradient (A: H_2O; B: ACN)	+ESI with MRM	0.3 - 100 ng/mL	Stable-isotope internal standards (2); SPE shown to significantly reduce matrix effects in tissue
Tai and Welch, 2005	17β-E2	Human serum	SPE with C18 Sep-Pak and STRX followed by	Zorbax Eclipse XDB-C18 (2.1x150mm, 5 micron)	Gradient (A: H_2O; B: ACN)	+ESI with MRM	~1.3 - 3.9 ng/mL	Stable-isotope internal standard; reference procedure

			derivatization with DC					for E2 from serum developed by NIST
Tai, 2006	P	Human serum	LLE with hexane	Zorbax Eclipse XDB-C18 (2.1x 150mm, 5 micron)	Gradient (A: 0.5% CH_3COOH in H_2O; B: MeOH)	+ESI with MRM	~18 - 54 ng/mL	Stable-isotope internal standard; reference procedure for P from serum developed by NIST
Tai, 2007	T	Human serum	SPE with C18 Sep-Pak and LLE with hexane	Zorbax Eclipse XDB-C18 (2.1x 150mm, 5 micron)	Isocratic (A: H_2O; B: ACN 71:29)	+ESI with MRM	~19 - 58 ng/mL	Stable-isotope internal standard; reference procedure for T from serum developed by NIST
Thieme, 2013	T	Saliva (1mL)	Buffered and extracted (LLE) with MTBE	Kinetex C18 (2.1x100mm, 2.6 micron)	Gradient (A: H_2O; B: ACN both with 2mM AA)	+ESI with MRM	50 - 5000 pg/mL	Stable-isotope internal standard; method applied to therapeutic drug monitoring (TDM) of T after transdermal administration
Voegel, 2018	Total of 12 steroids, including T and P	Human fingernails (0.5-10mg)	LLE with ethyl acetate or SPE with Oasis HLB or Ev	Kinetex XB-C18 (2.1x 50mm, 2.6 micron)	Gradient (A: H_2O; B: MeOH both with 0.2mM NH_4F)	+ESI with MRM	5 - 1000 pg/mg	Stable-isotope internal standards; 6 of the steroid analytes

			olute Express ABN					were quantifiable in authentic nail samples
Wang, 2015	Total of 6 free and conjugated estrogens, including 17β-E2	Human serum (100μL)	β-glucuronidase treatment followed by LLE with MTBE and derivatization with NMPS	Waters BEH 130 C18 (0.15x100 mm, 1.7 micron)	Gradient (A: H_2O; B: ACN both with 0.1% FA)	+ESI with SRM	0.5 - 200 pg/mL	Stable-isotope internal standards; novel derivatization technique results in quaternized analytes
Wooding, 2015	17β–E2, E1, T	Human serum (100μL)	96-well SLE+ with dichloromethane elution	Kinetex C18 (3x100mm, 2.6 micron)	Gradient (A: H_2O; B: MeOH with 10% NH_4OH in ACN post-column)	+/- ESI with MRM	0.001 - 1 ng/mL	Stable-isotope internal standards; LCMS assay compared to ELISA, finding that quantification of estrogens is more accurate using MS
Xu, 2005	Total of 15 endogenous estrogens, including 17β–E2	Human urine (0.5mL)	β-glucuronidase treatment followed by LLE with DCM and derivatization with DC	Synergi Hydro-RP (2.0x150mm, 4 micron)	Gradient (A: H_2O with 0.1% FA; B: MeOH)	+ESI with SRM	0.04 - 38.4 ng/mL	Stable-isotope internal standards; assay used in a study of postmenopausal women to investigate

								relationship between estrogen metabolism and breast cancer risk
Xu, 2007	Total of 15 endogenous estrogens, including 17β–E2	Human serum (500μL)	β-glucuronidase treatment followed by LLE with DCM and derivatization with DC	Synergi Hydro-RP (2.0x150mm, 4 micron)	Gradient (A: H_2O with 0.1% FA; B: MeOH)	+ESI with SRM	8 - 160 pg/mL	Stable-isotope internal standards; assay used in a study of postmenopausal women to investigate relationship between estrogen metabolism and breast cancer risk
Xu, 2016	T, androstene-dione, DHEA	Human serum (100μL)	PP with MeOH	Luna C18 (2.1x100mm, 5 micron)	Gradient (A: H_2O; B: MeOH)	+APCI with MRM	1-25 nM	Stable-isotope internal standards; blank serum double charcoal stripped using Chrom-matrix C18 columns; study used to

								establish reference androgen ranges in Chinese adults
Yamashita, 2007	17β-E2 and E1	Human serum (500µl - 1mL)	LLE (diethyl ether) followed by derivatization with picolinoyl chloride and SPE (Bond Elut C18)	Cadenza-CD C18 (3.0x150mm, 3 micron)	Gradient (A: H_2O with 0.1% CH_3COOH; B: ACN-MeOH)	+ESI with MRM	0 - 1000 pg/mL	Stable-isotope internal standards; SPE used after derivatization to remove excess agent
Yoo, 2019	17β-E2, T, DHEA	Mouse adipose tissue (100mg)	PP with ACN followed by LLE with hexane	Ascentis Express RP-Amide (2.1x100mm, 2.7 micron)	Gradient (A: H_2O; B: MeOH each with 0.1% FA)	+APCI with MRM	2 - 100 femto-mole	Stable-isotope internal standards; extraction enabled differentiation between free and sulfated steroids
Yuan, 2019	Total of 7 steroids, including T, P, and 17β-E2	Human serum (100µL)	LLE with MTBE followed by derivatization with DC	Acquity BEH C18 (2.1x 10mm, 1.7micron)	Gradient (A: H_2O; B: MeOH each with 0.2% FA)	+ESI with MRM	0.01 - 10ng/mL	Stable-isotope internal standards; Assay applied to a study of link between sex hormone levels and coronary artery disease in males

Zhang, 2008	P	Human plasma (400μL)	SPE with Oasis HLB	Symmetry C18 (2.1x 50mm, 3.5 micron)	Gradient (A: 5% MeOH in H_2O; B: MeOH each with 2mM AA and 0.1% FA)	+ESI with MRM	2 - 400 ng/mL	Medroxy-progesterone acetate used as internal standard; applied to a study in pregnancy patients, male plasma interferants investigated
Zhang, 2011	17β-E2, T	H295R cells	LLE with DCM followed by derivatization with DC	Zorbax SB-C18 (4.6x 50mm, 1.8 micron)	Gradient (A: H_2O; B: ACN each with 0.1% CH_3COOH)	+API with MRM	10-2500 pg/mL	Stable-isotope internal standards; evaluated for inclusion in EPA Endocrine Disruptor Screening Program
Zheng, 2019	Total of 13 steroids, including T, P, and 17β-E2	Human urine (0.5mL)	SPE with Oasis HLB	Acquity BEH C18 (2.1x 10mm, 1.7micron)	Gradient (A: H_2O with 0.1% FA; B: ACN)	+/- HESI with PRM	n/a	Reference steroids used to investigate hormones profiles in male & female patients; resulted in 80 and 107 characterized or tentatively identified

Zhou, 2020	Total of 14 steroids, including T, P, and 17β-E2	Human urine (1mL)	LLE with MTBE: ethyl acetate (5:1)	Acquity BEH C18 (2.1x 10mm, 1.7micron)	Gradient (A: H_2O with 0.1% FA; B: ACN)	+ESI with MRM	0.2 - 20000 ng/mL	Stable-isotope internal standards (4); assay applied to urine profiling of steroids with short run time

VITA

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Publications:

Gravitte A, Archibald T, Cobble A, Kennard B, Brown S. Liquid chromatography-mass spectrometry applications for quantification of endogenous sex hormones. Biomed Chromatogr. 2021 Jan;35(1):e5036. doi: 10.1002/bmc.5036. Epub 2020 Dec 23. PMID: 33226656.

Berry A*, Hall JV. The complexity of interactions between female sex hormones and *Chlamydia trachomatis* infections. Curr Clin Microbiol Rep. 2019 Jun;6(2):67-75. doi: 10.1007/s40588-019-00116-5. Epub 2019 May 11. PMID: 31890462; PMCID: PMC6936955.

Kruppa MD, Jacobs J, King-Hook K, Galloway K, **Berry A***, Kintner J, Whittimore JD, Fritz R, Schoborg RV, Hall JV. Binding of Elementary Bodies by the Opportunistic Fungal Pathogen *Candida albicans* or Soluble β-Glucan, Laminarin, Inhibits *Chlamydia trachomatis* Infectivity. Front Microbiol. 2019 Jan 14;9:3270. Doi: 10.3389/fmicb.2018.03270. PMID: 30692972; PMCID: PMC6339894.

Presentations:

Berry A*, Kintner J, Hall, JV. *Estrogen receptors affect Chlamydia muridarum infection in mice.* Poster presentation. Chlamydia Basic Research Society Meeting in Seattle, WA, March 2019.

Yakoob H, Liu C, Grimm M, Slade J, **Berry A***, Leonard C, Kintner J, Schoborg R. *The Human Immunodeficiency Virus (HIV) drugs dolutegravir, elvitegravir, and bictegravir inhibit Chlamydia trachomatis and C. muridarum in cell culture*. Poster presentation. Chlamydia Basic Research Society Meeting in Seattle, WA, March 2019.

Berry A*, Kintner J, Hall, JV. *Estrogen receptors affect Chlamydia muridarum infection in mice.* Oral presentation. Appalachian Student Research Forum, April 2019.

Berry A*. *Chlamydial interactions with the female sex hormones estrogen and progesterone and their receptors*. ETSU Department of Biomedical Sciences Internal Seminar Series, September 2019.

Huskey B, **Berry A***, Kintner J, Hall JV. *Determining the effects of the inhibition of Chlamydia muridarum host cell exit mechanisms.* Quillen College of Medicine Research Symposium. September 2019.

Honors and Awards:

1st Place in Graduate Student-Doctoral Physiology, Biology division at the 2021 Appalachian Student Research Forum. Estrogen treatment of ovariectomized mice protects against *Chlamydia muridarum* infection.

ETSU 3 Minute Thesis Finalist. Spring 2021. Estrogen signaling affects chlamydial infection in mice

ETSU School of Graduate Studies Graduate Student Research Grant. Spring 2019. Determining the effects of *Chlamydia muridarum* host cell exit mechanisms.

Chlamydia Basic Research Society Travel Award. Spring 2019.

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