IMPACT OF EARLY WEANING, BIOLOGICAL SEX, AND CASTRATION ON ILEAL MUCOSAL TRANSCRIPTOMIC RESPONSES TO LPS CHALLENGE IN PREPUBERTAL PIGS

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ABSTRACT

Adverse experiences that occur early in life, known as early-life adversity (ELA), include factors such as the loss of a parent, exposure to violence, malnutrition, and infections; can disrupt normal developmental processes and significantly increase the risk of diseases later in life. Biological sex is another factor influencing disease risk, with evidence indicating that males and females react differently to ELA. Notably, females tend to be more vulnerable to the adverse long-term effects of ELA. In previous studies conducted by our group, early-weaning stress in pigs—a model for ELA—was linked to a hyperactive enteric nervous system, compromised intestinal barrier function, functional diarrhea, and chronic low-grade intestinal inflammation later in life. These effects were much more pronounced in female pigs compared to castrated male pigs. Still, the exact molecular mechanisms associated with this sexual dimorphism are still not well elucidated. Therefore, the objectives of the present study were to compare how female, male, and castrated male pigs weaned at either 15 days (early weaning condition) or 28 days (late weaning condition) respond to a lipopolysaccharide (LPS) challenge later in life (70-day-old), by evaluating the transcriptome of the ileum mucosa. By comparing samples from female and male pigs, we want to assess the effect of biological sex on the immune response, and by comparing male and castrated male pigs, we want to evaluate how castration status impacts the immune response.

I dedicate this work to my mother, Denise, whose strength inspires me, and to all my friends.

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CHAPTER 1: A LITERATURE REVIEW ON SEX DIFFERENCES IN NEUTROPHIL BIOLOGY

1.1.ABSTRACT

Males are more susceptible to infectious diseases and experience higher mortality rates compared to females. However, the molecular and cellular mechanisms behind this increased risk remain poorly understood. Previous research from our group indicated that female pigs have a higher neutrophil-to-lymphocyte ratio than males and that castration of male pigs impairs neutrophil migration. In this review, we explore sex differences and the effect of sex hormones on neutrophil kinetics and effector functions in both human and animal models. In adult human subjects, there are sex differences in the transcriptional profiles of neutrophils. Females show increased expression of interferon-stimulated genes, while males exhibit heightened expression of genes related to chemotaxis, granule formation, and cellular respiration, indicating a more immature phenotype compared to females. Androgens enhance granulopoiesis, leading to greater neutrophil production in males than in females. They also stimulate the expression of cytokines, chemokines, and adhesion molecules, which promote neutrophil interactions with the blood vessel wall and facilitate their migration. However, despite this stronger neutrophilic response, males demonstrate reduced pathogen clearance, possibly due to diminished myeloperoxidase activity and impaired phagocytic and degranulation functions associated with androgen exposure. Overall, delayed clearance and persistence of inflammatory signaling result in tissue damage. In contrast, estrogens—produced at higher levels in adult females—appear to exert protective effects by inhibiting excessive neutrophil activation, thereby reducing tissue damage and disease severity. These sex differences in neutrophil function may contribute to the sex bias observed in both infectious and non-infectious diseases, such as cardiovascular diseases, neurodegenerative disorders, and certain cancers. Understanding these sex differences in neutrophil function is crucial

for developing personalized therapeutic strategies aimed at improving infection outcomes and managing inflammatory diseases.

1.2.INTRODUCTION

Females mount a more robust inflammatory response than males, which provides an advantage in clearing infections. This difference was evident during the COVID-19 pandemic when females had lower mortality rates than males (Mourosi et al., 2022). However, this heightened immune reactivity also makes females more susceptible to immune-mediated disorders. For example, females account for more than 70% of all reported autoimmune disease cases (Ramos-Casals et al., 2015) and are 2.2 times more likely than males to experience drug-induced anaphylaxis (Dhopeshwarkar et al., 2019). Thus, while a more robust immune response enhances pathogen defense, it also increases the risk of immune dysregulation in females.

These sex differences in the immune response and disease outcomes have been attributed to different factors, including genetic differences associated with the sex chromosomes, but also the influence of sex hormones that can impact the immune system at different stages of development. Females have two copies of the X chromosome, which contains approximately 50 genes involved in immune regulation (Dunn et al., 2024; Forsyth et al., 2024; Klein & Flanagan, 2016). Although one X chromosome undergoes inactivation early in embryonic development, some immune-related genes escape inactivation and are expressed at higher levels. For example, toll-like receptor 7 (*TLR7*), toll-like receptor 8 (*TLR8*), and TLR adaptor interacting with endolysosomal SLC15A4 (*TASL*)— important in antiviral defense—are more highly expressed in females, potentially contributing to their enhanced immune response and greater resistance to infections compared to males (Forsyth et al., 2024).

In addition, hormones produced in the gonads, such as estrogens and androgens, regulate

immune functions at different stages of development. Estrogens are considered pro-inflammatory and are produced in high quantities by the ovaries in adult females, while androgens, which are generally regarded as immunosuppressive, are mainly produced in the testis by males. However, these effects can be highly context-dependent and vary based on cell type (Dunn et al., 2024; Klein & Flanagan, 2016). Interestingly, sex steroids also influence immune function before puberty. The perinatal androgen surges, which occur exclusively in males and are known to regulate sex differentiation, also affect immune cells, helping to establish sex differences in immune function that persist into adulthood (Ghosh et al., 2021; Mackey et al., 2020).

In this context, neutrophils—the first cells to migrate to the site of infection—are regulated differently in females and males. Neutrophils are important during inflammation, pathogen clearance, and tissue repair. Previous studies have demonstrated that female pigs (*Sus scrofa*) exhibit a stronger systemic immune response than their male counterparts, as evidenced by a higher neutrophil-to-lymphocyte ratio, particularly following early-life stressors such as early weaning stress (Fardisi et al., 2023). In addition, castrated male pigs have compromised neutrophil migration, which further highlights the role of sex hormones in shaping neutrophil function. However, little is known about the mechanisms driving these sex differences and how castration, a common practice in the pork industry, impacts the immune system.

Developing a better understanding of the mechanisms driving sex differences in the immune system in pigs is important, with implications for both biomedical research and animal health. First, the pig immune system shares many similarities with humans, making pigs a valuable preclinical model for studying sex differences in the immune system and their impact on disease susceptibility and treatment outcomes (Bréa et al., 2012; Meurens et al., 2012). Second, pork is one of the most widely consumed meats globally, and infectious diseases remain a major challenge

in swine production (VanderWaal & Deen, 2018). A better understanding of the mechanisms underlying sex differences in neutrophil function could inform strategies to enhance disease resistance and reduce mortality, ultimately improving animal welfare and production efficiency. Finally, castration is a routine practice in the swine industry, performed to prevent boar taint, a meat quality problem linked to the accumulation of androstenone in the carcass, and few studies have explored how castration impacts immune function.

The objectives of the present review are to explore sex differences in neutrophil kinetics and effector functions and describe how sex steroids mediate these differences in both human and animal models.

1.3.SEX DIFFERENCES IN NEUTROPHIL PHENOTYPE

Neutrophils are the most abundant circulating white blood cells (WBCs) and play a crucial role in the innate immune response. Widely known for their characteristic segmented nuclei, short lifespan, and high motility, neutrophils are the first cells to respond to the chemokines generated at sites of injury or infection. Upon arrival, they transmigrate the vessel wall and deploy various effector functions, including chemotaxis, phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs), to neutralize pathogens. Their rapid and coordinated response to danger signals is critical for an effective immune defense, and disruptions in neutrophil function can result in undesirable health outcomes.

Deficient neutrophil recruitment to the site of infection and impaired phagocytic and oxidative burst capacity compromise pathogen clearance capacity, increasing susceptibility to bacterial and fungal infections and delaying wound healing (Marciano et al., 2015; Chonchol, 2006; Roos et al., 2023). Conversely, prolonged neutrophil recruitment, excessive activation, and accumulation at sites of inflammation can result in severe tissue damage and are central to the

pathogenesis of infectious diseases, such as sepsis, as well as chronic inflammatory diseases, such as Alzheimer's, atherosclerosis, inflammatory bowel disease, and arthritis (Soehnlein et al., 2017). Therefore, sex differences in neutrophil function may underlie and contribute to the sex biases observed in these diseases.

Emerging evidence suggests that sex differences exist in human neutrophil populations, influencing their activation, chemotaxis, and effector functions at injury sites. A study by Wang et al. (2024) utilized single-cell RNA sequencing to investigate these differences in adult human subjects. They identified five distinct subpopulations of neutrophils and found that men had a higher proportion of inflammatory neutrophils while women exhibited a greater number of unconventional neutrophils. In contrast, a similar study by Gupta et al. (2020) reported no differences in the proportions of neutrophil subpopulations between men and women. However, it did uncover sex differences in gene expression within a single subpopulation. Both studies reported that female neutrophils demonstrate higher expression levels of type I interferon-stimulated genes, a finding that was also supported by Er-Lukowiak et al. (2023).

Further functional analysis using immunofluorescence demonstrated that transcription factors associated with type I interferon signaling exhibited stronger activity in neutrophils derived from adult females than adult males. In neutrophils from female subjects, interferon regulatory factor 9 (IRF9), phosphorylated IRF3, and the p65 subunit of nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) were predominantly localized in the nucleus, which suggests that these transcription factors were more readily able to act on interferon-stimulated response elements. In contrast, neutrophils from males primarily displayed these proteins in the cytosol (Gupta et al., 2020). Supporting these results, neutrophils from adult female subjects stimulated *ex vivo* with phorbol myristate acetate (PMA) had higher expression of viperin, an interferon-

stimulated gene, than adult male subjects (Er-Lukowiak et al., 2023).

However, it is important to notice that the sex bias observed in interferon signaling pathways is not limited to neutrophils. Similar sex differences have been reported in various immune cells from human subjects, including classical monocytes, non-classical monocytes, naïve B cells, naïve CD4+ T cells, naïve CD8+ T cells, activated naïve CD4+ T cells, and activated naïve CD8+ T cells (Schmiedel et al., 2018).

Another functional distinction between neutrophils from female and male subjects lies in their bioenergetics profile. Neutrophils from adult male subjects have higher mitochondrial activity, as measured by oxygen consumption rate, higher mitochondrial DNA content, and mitochondrial mass than neutrophils from females (Gupta et al., 2020). Consistent with these findings, Wang et al. (2024) reported higher expression of cellular respiration genes in male neutrophils. Mitochondrial activity is highest in immature neutrophils, particularly less segmented neutrophils found in bone marrow (Rice et al., 2018). These findings suggest that male neutrophils exhibit a more immature phenotype than female neutrophils (Gupta et al., 2020). Furthermore, since mitochondrial respiration is crucial for sustaining oxidative burst and reactive oxygen species (ROS) production in glucose-limited environments—such as those found in chronic inflammation and the tumor microenvironment (Rice et al., 2018)—these metabolic differences may contribute to the observed sex bias in these disorders.

Sex differences in neutrophil interferon signaling and bioenergetics have been observed in adults, but not in prepubertal pediatric subjects. This indicates that such differences may be driven by post-pubertal changes in sex hormone levels rather than by differences in sex chromosome composition (Gupta et al., 2020). To explore the potential role of chromosome complement in regulating the expression of interferon-stimulated genes in neutrophils, gene expression among

females (XX), males (XY), and males with Klinefelter syndrome (XXY). The study found no significant differences between the two male groups, while females exhibited higher expression levels than males in both XY and XXY groups (Gupta et al., 2020). Additionally, Gupta et al. (2020) investigated the impact of estradiol treatment on male neutrophils. Administering estradiol (200 pg/mL) induced a bioenergetic phenotype in male neutrophils resembling female neutrophils. This finding supports the notion that sex hormones modulate neutrophil metabolism and function (Gupta et al., 2020).

In summary, these studies consistently report sex differences in neutrophils isolated from adult human subjects; while female neutrophils exhibit stronger interferon signaling, male neutrophils have a more immature profile. Sex hormones play a key role in shaping neutrophil metabolism and immune responses, which may contribute to sex-based differences in susceptibility to and severity of chronic inflammation, cancer, and autoimmune diseases. However, a limitation of studying human neutrophils is their short lifespan, as they undergo apoptosis within hours of isolation, limiting the feasibility of complex experimental approaches, such as genetic modifications or neutrophil kinetics during inflammation (Burn et al., 2021). To overcome these challenges, animal models, such as rodents and pigs, provide valuable alternatives for investigating the molecular mechanisms underlying sex differences in neutrophil function and their role in inflammation and pathogen clearance.

1.4.SEX DIFFERENCES IN NEUTROPHIL RECRUTIMENT

Neutrophils are produced from progenitor cells located in the bone marrow, which serves as the primary reservoir of neutrophils in the body. In humans, neutrophils account for 50-70% of the immune cells found in the blood (Hidalgo et al., 2019). Under normal physiological conditions, the bone marrow releases over 100 billion neutrophils daily. However, this production can increase

up to tenfold in response to injury or infection (Mayadas et al., 2014). This remarkable capacity for rapid mobilization, along with the significant number of neutrophils that arrive at infection sites, underscores the critical role these cells play in immune surveillance and the inflammatory response. Accumulating evidence from both human and animal studies indicates that males tend to recruit a greater number of neutrophils to the site of infection (Table 1.1), and these sex differences are associated with different mechanisms.

The proliferation, differentiation, and maturation of neutrophils primarily occur in the bone marrow and are regulated by granulocyte colony-stimulating factor (G-CSF). However, sex hormones and their receptors significantly influence this process. Male mice have greater numbers of neutrophil precursors and mature neutrophils in the bone marrow compared to female and castrated male mice (Tang et al., 2020). Corroborating these findings, Madalli et al. (2015), using a mesentery ischemia-reperfusion injury model, demonstrated that the greater intensity of neutrophil recruitment in males is linked to differences in G-CSF expression in both the site of injury and the bone marrow. Ischemic reperfusion injury significantly increased G-CSF expression 30 minutes after the injury in males but not females. Furthermore, male rats exhibited much higher G-CSF expression levels at 30 minutes and 2 hours after the stimulus than female rats (Madalli et al., 2015).

Androgens, through the androgen receptor (AR), also regulate granulopoiesis. In experiments using a knockout mouse model for the androgen receptor (ARKO), a considerable reduction in the percentage of circulating neutrophils was observed in androgen receptor knockout animals ($3.7 \pm 2.2\%$ in ARKO mice vs. $22.6 \pm 7.2\%$ in wild-type mice, $p < 0.001$). Interestingly, androgen supplementation restored normal numbers of neutrophils in castrated males but not in ARKO or testicular feminization (Tfm) mice, a model with an inactivated AR due to a nonsense

mutation. Furthermore, Chuang et al. (2009) demonstrated that the androgen receptor regulates terminal differentiation of neutrophils. Androgen receptor knockout did not interfere with the number of early progenitors, such as common myeloid progenitor cells and granulocyte-macrophage progenitor cells. Still, it was associated with a significant reduction in the number of committed neutrophil precursors, such as neutrophil myelocytes and neutrophil metamyelocytes (Chuang et al., 2009).

Additionally, there are sex differences in chemokine expression in response to inflammatory stimuli. Male rats had increased expression of C-X-C chemokine receptor type 2 (*Cxcr2*) – a chemokine receptor that regulates neutrophil migration and recruitment – and its ligand C-X-C motif chemokine 5 (*Cxcl5*) in the bone marrow at both 30 minutes and 2h after injury stimulus; this response was not seen in female rats (Madalli et al., 2015). Moreover, exogenous administration of *Cxcl5* stimulated transcription of G-CSF and *Cxcr2* in both female and male rats (Madalli et al., 2015). In agreement with these findings, androgens stimulate the expression of chemokines, such as *Ccl2*, *Ccl3*, *Ccl4*, *Cxcl1*, *Cxcl2 Cxcl4*, and *Cxcl7* (Scalerandi et al., 2018; Chuang et al., 2009). The higher production of androgens in males, especially after puberty, may underly the increased migratory capacity of neutrophils in male animals.

Sex differences are apparent in the expression of adhesion molecules, especially under stress conditions. Estrogens inhibit adhesion molecule expression, reducing the interaction between leukocytes and blood vessels. Inflammatory stimuli such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) induce greater neutrophil migration in sexually mature male mice compared to female mice. This difference is associated with a significant upregulation of P-selectin expression on the endothelial cells of male mice in response to these cytokines, but not in females (Villar et al., 2011). Notably, estrogens, found in higher amounts in sexually mature females than

males, inhibit the expression of P-selecting in endothelium cells (Villar et al., 2011) and the expression of β2 integrin and L-selectin in the surface of neutrophils (Nadkarni et al., 2011). Corroborating these studies, *Cxcl5* induced significantly higher expression of β2 integrin (CD11b) and ICAM-1 in male rats compared to female rats (Madalli et al., 2015).

Under normal homeostatic conditions, neutrophils released from the bone marrow enter the peripheral vasculature, where they can exist in two distinct forms: a freely circulating pool and a marginated pool, the latter consisting of neutrophils adhered to the endothelium via adhesion molecules. While neutrophils are typically present in low numbers within tissues, the spleen, lungs, and liver are known to accumulate a large number of marginated neutrophils and form considerable extramedullary pools (Hidalgo et al., 2019; Ng et al. 2019). The significance of these extramedullary pools is still not completely elucidated but their strategic anatomical position suggests a crucial role in immune defense.

In particular, the spleen is a relevant site of extramedullary granulopoiesis, especially under stress conditions, when the demand for neutrophils is higher, such as disseminated infection or autoimmune disorders (Guo et al., 2024). In healthy mice, neutrophil precursors account for 1.01% of the total neutrophil population in the spleen, compared to the 3.55% of neutrophil precursors found in the bone marrow. These findings corroborate the importance of the spleen in extramedullary granulopoiesis in both homeostatic and stress conditions (Grieshaber-Bouyer et al., 2021). However, the spleen microenvironment differs from the bone marrow microenvironment, and neutrophils that mature in the spleen can interact with other immune cells and acquire unique phenotypes, contributing to the heterogeneity of neutrophil populations. Moreover, neutrophils that develop in the spleen are thought to potentially play a role in the pathogenesis of infectious and inflammatory diseases (Guo et al., 2024; Sengupta et al., 2020).

There are differences in the spleen immune cell population of females and males, including lymphocytes, monocytes, and neutrophils (Ghosh et al., 2021; Menees et al., 2021;Kay et al., 2015). Using the four-core genotype model, Ghosh et al. (2021) showed that variations in the number of spleen lymphocytes are primarily driven by sex hormones rather than chromosome complement. These differences are established early in life due to surges of androgens during the perinatal period (Ghosh et al., 2021). Although Ghosh et al. (2021) did not assess neutrophil counts directly, lymphocyte populations can modulate neutrophil function, and sex differences in lymphocyte numbers may contribute to sex-specific neutrophil responses during inflammation (Scotland et al., 2011). Moreover, age and immunosenescence also influence sex differences in immune cell populations in the spleen. Young but sexually mature male mice, around 2–3 months of age, have a larger pool of splenic neutrophils than female mice within the same age range. However, as mice age and undergo immunosenescence (18–22 months), neutrophil counts in the spleen decrease in males but increase in females. These findings are interesting and may have translational relevance for studies associated with degenerative diseases, which are more common in the older population and are known to have a sex bias (Menees et al., 2021).

Similarly, in a study using a zymosan-induced peritonitis model, Kay et al. (2015) found that adult male mice (2-3 months old) possess a larger pool of splenic neutrophils than female mice under baseline conditions. This larger pool can be readily mobilized in males in response to stimuli, resulting in a more significant number of neutrophils recruited to the injury site. Furthermore, female and male mice have different rates of neutrophil recruitment and release from the spleen during the inflammatory response. While males tend to have higher mobilization rates, female mice have higher retention of neutrophils in the spleen. To illustrate this, female mice that received an intraperitoneal injection of zymosan had double the number of neutrophils in the spleen

compared to females in the control group, while the number of spleen neutrophils in males remained constant (Kay et al., 2015).

Though the marginated pools of neutrophils found in the lungs and liver are not as well studied as the spleen pool, their strategic anatomical positioning suggests a crucial role in immune defense. The lungs represent the largest interface with the external environment, and the liver, receiving blood directly from the gastrointestinal tract via the portal circulation, are both points of potential pathogen encounter. The high accumulation of marginated neutrophils in these organs likely increases the chances of encountering inflammatory stimuli. These neutrophils are thought to act as rapidly mobilizable cells, enabling a swift and effective response to inflammatory signals and thereby enhancing host defense against infections (Haynes et al., 2024; Hidalgo et al., 2019).

Although sex differences in the lung and liver neutrophil populations under non-stimulated conditions were not reported, males demonstrate a higher rate of neutrophil recruitment to these organs compared to females during inflammatory responses. Furthermore, the severity of diseases affecting the lung and liver—such as acute respiratory distress, liver metastasis, and hepatic amebiasis—tends to be greater in males (Er-Lukowiak et al., 2023; Tang et al., 2022; Cochi et al., 2016; Lotter et al., 2006). These disparities are partially influenced by AR signaling. The liver is a preferred site for the metastasis of solid tumors. In humans, males are more susceptible to liver metastasis for tumors such as colorectal cancer, lung cancer, pancreatic cancer, melanoma, and stomach cancer (Tang et al., 2020). In the mouse model, sex differences in neutrophil recruitment contribute to sex bias in liver metastasis. In addition, castration, AR knockout ($Ar^{-/-}$), and treatment with an antagonist of the AR have been shown to reduce liver metastasis in males to levels similar to that of female mice (Tang et al., 2020).

Similarly, males are more susceptible to respiratory distress (Cochi et al., 2016). In a mouse

model of airway hyperresponsiveness to ozone, while females have increased lung dysfunction at 12h after exposure to ozone, males develop a more persistent response and have higher respiratory resistance and elastance by 48 and 72 h after exposure (Birukova et al., 2019). Moreover, reducing the intensity of AR signaling through castration or treatment with flutamide, an AR antagonist, has been shown to lessen airway hyperresponsiveness to ozone and decrease cellular recruitment to the lungs in male mice (Osgood et al., 2019). Complementing these findings, male rats had increased expression of Cxcl5 in the lungs compared to female rats, which contributed to the greater accumulation of neutrophils in the lungs, measured by myeloperoxidase (MPO) activity, even when the lung was not the primary site of inflammation (Madalli et al., 2015).

In summary, sex differences in neutrophil recruitment are primarily influenced by sex hormone levels. Androgens augment granulopoiesis and the expression of chemokines, which ultimately leads to greater production of neutrophils in the bone marrow and greater recruitment of neutrophils to the site of infection in sexually mature males. In contrast, estrogens inhibit the expression of adhesion molecules on both the endothelium and the surface of neutrophils, making the process of transmigration—a critical step in neutrophil recruitment—less efficient. Additionally, males have larger pools of splenic neutrophils, a cell population that can be quickly mobilized during inflammation, whereas females tend to retain more neutrophils in the spleen during such responses. The liver and lungs are key sites for the accumulation of marginated neutrophils, and the higher recruitment of neutrophils to these organs contributes to the male bias observed in respiratory and hepatic diseases.

1.5.SEX DIFFERENCES IN NEUTROPHIL ACTIVATION AND EFFECTOR FUNCTIONS

Despite a heightened activation and mobilization of neutrophils to sites of injury or

infection, sexually mature males demonstrate impaired clearance of pathogens compared to females. In a mouse model of *Entamoeba histolytica* intrahepatic infection, female mice successfully eliminated the pathogen by day 3 post-infection. In contrast, male mice exhibited a stronger inflammatory response, characterized by increased immune cell infiltration. However, this exacerbated response was insufficient to clear the infection, and male mice continued to harbor viable parasites even 14 days post-infection. A similar outcome was observed in an intraperitoneal infection model, where males showed reduced bacterial clearance after being inoculated with Group B Streptococcus compared to females (Scotland et al., 2011).

The greater efficiency of females in eliminating pathogens and clearing infections may be associated with higher immune activation under baseline conditions. For instance, under non-stimulated conditions, female mice and rats showed significantly higher leukocyte counts in the peritoneal and pleural cavities compared to males ($31 \pm 2.3 \times 10^5$ vs. $16 \pm 1.7 \times 10^5$, measured in peritoneal fluid) (Scotland et al., 2011). Notably, female resident peritoneal macrophages showed higher TLR2, TLR3, TLR4, and MyD88 expression than their male counterparts, enhancing their ability to detect and respond to infections efficiently through improved pathogen recognition and phagocytic activity of these cells. The differential regulation of these molecules between female and male subjects is largely associated with levels of sex hormones (Yang et al., 2020; Scotland et al., 2011; Rettew et al., 2009; Rettew et al., 2008).

Moreover, reduced oxidative burst activity in male subjects may contribute to male susceptibility in clearing the infection. Human neutrophils derived from male subjects had reduced myeloperoxidase (MPO) activity in neutrophils compared to female neutrophils. This enzyme is critical for the oxidative burst response, converting hydrogen peroxide (H_2O_2) and chloride ions (Cl^-) into hypochlorous acid (HOCl), a potent antimicrobial agent (Emokpae & Mrakpor, 2016;

Kabutomori et al., 1999). Complementing these findings, testosterone exposure was linked to impaired bactericidal and reduced MPO activity (Scalerandi et al., 2018). Notably, testosterone-treated male mice maintained higher numbers of live *Escherichia coli* than control animals (Scalerandi et al., 2018).

Conversely, mice exposed to testosterone had increased neutrophil protein content of NADPH Oxidase 1, NADPH Oxidase 2, and NADPH Oxidase 3 (Chignalia et al., 2015). These enzyme subunits convert oxygen into superoxide anion (O_2^-), an earlier step in the oxidative burst response. Overall, this suggests that while testosterone enhances the potential for reactive oxygen species production via NADPH oxidase, the reduction in MPO activity may compromise the overall effectiveness of the neutrophil antimicrobial response, as this last enzyme catalyzes the production of a more potent antimicrobial compound.

Androgens may also underlie male susceptibility to infection and sepsis through inhibition of phagocytosis and degranulation function. Testosterone exposure was associated with delayed clearance and increased tissue damage. In a model of urinary tract infection, female mice that received testosterone cypionate prior to infection exhibited persistent bacterial loads and developed more severe upper (pyelonephritis) and lower (cystitis) urinary tract infections compared to the untreated group despite increased cellular infiltration (Hreha et al., 2024). Androgen treatment was linked to impaired neutrophil maturation and reduced phagocytic and degranulation capacity, an effect that was particularly pronounced in the kidney microenvironment (Hreha et al., 2024). In the kidney, androgenized females developed a distinct neutrophil subpopulation, characterized as aged (Cd49d+) and immature (Cd101-). This unique population accounted for 40% of the kidney neutrophils and exhibited a more intense reduction in the capacity for phagocytosis and degranulation (Hreha et al., 2024).

Moreover, higher levels of testosterone were also linked to higher production of anti-inflammatory cytokines (IL-10 and TGFβ-1) and lower levels of pro-inflammatory cytokines (IL-1β and TNF-α) in male mice neutrophils (Scalerandi et al., 2018). According to Scalerandi et al. (2018), testosterone treatment induces an anti-inflammatory neutrophil phenotype resembling the "N2" subtype observed in the cancer microenvironment. Likewise, Hofer et al. (2015) found that testosterone pretreatment delayed urethral healing in rats. However, the wound site showed increased production of both pro-inflammatory (TNF-α) and anti-inflammatory (IL-10 and TGFβ-1) cytokines, along with enhanced expression of growth factors involved in tissue repair, including FGF-10 and VEGF-α (Hofer et al., 2015).

In contrast, androgen receptor knockout mice demonstrated reduced activity of signal transducer and activator of transcription 3 (*Stat3*), lower expression of cytokines and chemokines, and showed greater susceptibility to infections compared to wild-type mice (Chuang et al., 2009). After receiving an intraperitoneal injection of *E. coli,* ARKO mice exhibited more severe infections and higher mortality rates, with a 100% mortality rate in androgen receptor knockout mice compared to 30% in wild-type mice (Chuang et al., 2009). These findings suggest that while testosterone may hinder certain aspects of neutrophil bactericidal activity, androgen signaling plays a critical role in neutrophil function, neutrophil cytokine production, and the overall efficacy of the immune response.

Estrogens also modulate neutrophil effector function. Deitch et al. (2006) reported that male neutrophils exhibit higher activation than female neutrophils following trauma-hemorrhagic shock or burn injury. This heightened activation was measured by increased cell surface expression of β2 integrin and enhanced oxidative burst capacity in response to stimulation of NADPH oxidase activity (Deitch et al., 2006). Interestingly, castration reduced these functions in males, while

ovariectomy enhanced them in females, suggesting that androgens and estrogens exert opposing effects on neutrophil activation and NADPH oxidase activity. Deitch et al. (2006) also demonstrated that these sex differences were not dependent on the estrous cycle. Notably, when estrogen levels are at their highest peak during the proestrus phase, NADPH oxidase activity in neutrophils is significantly reduced, compared to NADPH oxidase activity from neutrophils collected from females in the diestrus phase, lowest estrogen levels. These observations imply that estrogens can also suppress neutrophil function. Similarly, Békési et al. (2000) found that both androgens and estrogens inhibit superoxide production in human neutrophils, reinforcing the notion that sex hormones modulate neutrophil activity.

Supporting these findings, Zhang et al. (2019) demonstrated that estrogen exposure reduces neutrophil migration and oxidative burst by measuring NADPH oxidase activity in human neutrophils and differentiated neutrophil-like cells (DHL-60). Although estrogen treatment alone does not directly impact neutrophil migration, pretreatment with estrogen significantly inhibits chemotaxis and NADPH oxidase activity induced by the leukocyte chemoattractant peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), a potent activator of neutrophils. This inhibitory effect is mediated through the dephosphorylation of ERK kinases by MKP-2, a phosphatase induced by estrogen signaling, as ERK kinase phosphorylation is essential for neutrophil migration and NADPH oxidase activity (Zhang et al., 2019).

Moreover, another mechanism associated with the inhibitory effect of estrogens on neutrophil function is the suppression of calcium influx (Deitch et al., 2006). Neutrophils store calcium in the endoplasmic reticulum, and a continuous influx of calcium via the store-operated calcium entry (SOCE) pathway is essential for antimicrobial activities, including NADPH oxidase activity, phagocytosis, degranulation, and NETosis (Hann et al., 2020).

The inhibitory action of estrogen occurs via estrogen nuclear receptors (ERα/β) rather than the G protein-coupled estrogen receptor (GPER-1). Human neutrophils pretreated with ICI 182780, an antagonist of ERα/β, showed significantly reduced migration and superoxide production in response to fMLP. In contrast, pretreatment with G15, an antagonist of GPER-1, did not affect the neutrophil response to fMLP (Zhang et al., 2019).

Contrasting evidence suggests that G1, a specific agonist of GPER-1, promotes NADPH oxidase activity, extends neutrophil lifespan, and induces a proinflammatory phenotype. This phenotype is characterized by increased expression of interleukin-1β (IL1β), CXCL8, prostaglandin synthase 2 (PTGS2), suppressor of cytokine signaling 3 (SOCS3), and granulocyte colony-stimulating factor (G-CSF) (Rodenas et al., 2017). Similarly, exposure to estradiol and progesterone contributes to the increased lifespan of human neutrophils. This is associated with reduced activity of caspase 3, caspase 9, and mitochondrial cytochrome c release (Molloy et al., 2003).

In summary, these findings indicate that males experience impaired clearance of infections and delayed wound healing, contributing to sustained inflammatory signaling and continued recruitment of neutrophils, contributing to further inflammation and tissue damage. This impairment in efficiently clearing infection may be linked to a lower number of tissue-resident cells in males compared to females, such as macrophages, which play a crucial role in clearing infections. Exposure to testosterone, produced in higher levels in males, has also been associated with diminished neutrophil function, such as reduced MPO activity, a marker of neutrophil oxidative burst, as well as reduced phagocytic and degranulation capacities. Moreover, estrogens, produced in higher amounts in adult females, may exert a protective role against excessive neutrophil activation.

1.6.FINAL CONSIDERATIONS

Understanding sex differences in neutrophil function is crucial due to the role of these cells in clearing infection and wound healing, as well as their role in interacting with and regulating other immune cells. This understanding can advance personalized medicine and improve therapeutic strategies. Neutrophils are associated with various diseases that show sex biases, including infectious diseases, sepsis, autoimmune disorders, neurodegenerative and cardiovascular diseases, and cancer. Therefore, uncovering the mechanisms underlying sex differences in neutrophil biology could pave the way for more effective, sex-specific treatments. In adult humans, distinct neutrophil populations are observed between females and males. Female neutrophils exhibit stronger type I interferon signaling, while male neutrophils demonstrate higher expression of genes associated with primary granules, chemotaxis, and cellular respiration, suggesting a more immature phenotype in males. The absence of these differences in pediatric subjects indicates they are driven by sex hormones rather than sex chromosome composition. Sex hormones play a pivotal role in modulating neutrophil functions. Androgens enhance granulopoiesis and promote the expression of chemokines, cytokines, and adhesion molecules, facilitating neutrophil recruitment to infection sites in males. However, despite this heightened recruitment, males exhibit impaired pathogen clearance, potentially due to diminished myeloperoxidase activity and reduced phagocytic and degranulation capacities linked to androgen exposure. In contrast, estrogens appear to exert protective effects by inhibiting excessive neutrophil activation, thereby reducing tissue damage and inflammation in females.

1.7.TABLES

Table 1.1. Sex Differences in Neutrophil Recruitment Across Animal Models and Human Studies.

Reference	Animal	Model	Findings
(Kay et al., 2015)	C57BL/6 mice (8–11 wk)	Zymosan-induced peritonitis	Males had higher neutrophil and monocyte recruitment to the peritoneal cavity at 3h after zymosan injection
(Er-Lukowiak et al., 2023)	C57BL/6 mice (10–12 wk)	Hepatic amebiasis	Males had higher neutrophil recruitment, measured in the blood and liver, 3 days post-infection
(Madalli et al., 2015)	Wistar rats (8-10 wk)	Mesentery ischemia-reperfusion injury	Males had higher neutrophils recruitment, measured in the blood and peritoneal fluid, at 30 min and 2h of reperfusion
(Robert et al., 2011)	Sprague-Dawley rats (4-5 mo)	Renal ischemia-reperfusion injury	Males had more severe and persistent tissue damage and increased neutrophil infiltration in the kidney at 1-3 days post-injury
(Scotland et al., 2011a)	C57BL/6 mice (9-10 wk)	Peritonitis/pleurisy	Males had higher neutrophil recruitment to the peritoneal/ pleural cavity at 3h after challenge
Madalli et al. (2015)	Healthy humans	Cantharidin skin blister	Males had higher neutrophil recruitment
(Rathod et al., 2017)	Healthy humans	Cantharidin skin blister	No sex differences in neutrophil recruitment, higher expression of markers of neutrophil activation in males

REFERENCES

Birukova, A., Cyphert-Daly, J., Cumming, R. I., Yu, Y. R., Gowdy, K. M., Que, L. G., & Tighe, R. M. (2019). Sex modifies acute ozone-mediated airway physiologic responses. *Toxicological Sciences*, *169*(2), 499–510. https://doi.org/10.1093/toxsci/kfz056

Bréa, D., Meurens, F., Dubois, A. V., Gaillard, J., Chevaleyre, C., Jourdan, M. L., Winter, N., Arbeille, B., Si-Tahar, M., Gauthier, F., & Attucci, S. (2012). The pig as a model for investigating the role of neutrophil serine proteases in human inflammatory lung diseases. *Biochemical Journal*, *447*(3), 363–370. https://doi.org/10.1042/BJ20120818

Burn, G. L., Foti, A., Marsman, G., Patel, D. F., & Zychlinsky, A. (2021). The Neutrophil. In *Immunity* (Vol. 54, Issue 7, pp. 1377–1391). Cell Press. https://doi.org/10.1016/j.immuni.2021.06.006

Chignalia, A. Z., Oliveira, M. A., Debbas, V., Dull, R. O., Laurindo, F. R. M., Touyz, R. M., Carvalho, M. H. C., Fortes, Z. B., & Tostes, R. C. (2015). Testosterone induces leucocyte migration by NADPH oxidase-driven ROS- and COX2-dependent mechanisms. *Clinical Science*, *129*(1), 39–48. https://doi.org/10.1042/CS20140548

Chuang, K. H., Altuwaijri, S., Li, G., Lai, J. J., Chu, C. Y., Lai, K. P., Lin, H. Y., Hsu, J. W., Keng, P., Wu, M. C., & Chang, C. (2009). Neutropenia with impaired host defense against microbial infection in mice lacking androgen receptor. *Journal of Experimental Medicine*, *206*(5), 1181–1199. https://doi.org/10.1084/jem.20082521

Cochi, S. E., Kempker, J. A., Annangi, S., Kramer, M. R., & Martin, G. S. (2016). Mortality trends of acute respiratory distress syndrome in the United States from 1999 to 2013. *Annals of the American Thoracic Society*, *13*(10), 1742–1751. https://doi.org/10.1513/AnnalsATS.201512-841OC

Deitch, E. A., Ananthakrishnan, P., Cohen, D. B., Zhong Xu, D., Feketeova, E., & Hauser, C. J. (2006). Neutrophil activation is modulated by sex hormones after trauma-hemorrhagic shock and burn injuries. *Am J Physiol Heart Circ Physiol*, *291*, 1456–1465. https://doi.org/10.1152/ajpheart.00694.2005.-Recent

Dhopeshwarkar, N., Sheikh, A., Doan, R., Topaz, M., Bates, D. W., Blumenthal, K. G., & Zhou, L. (2019). Drug-Induced Anaphylaxis Documented in Electronic Health Records. *Journal of Allergy and Clinical Immunology: In Practice*, *7*(1), 103–111. https://doi.org/10.1016/j.jaip.2018.06.010

Dunn, S. E., Perry, W. A., & Klein, S. L. (2024). Mechanisms and consequences of sex differences in immune responses. In *Nature Reviews Nephrology* (Vol. 20, Issue 1, pp. 37–55). Nature Research. https://doi.org/10.1038/s41581-023-00787-w

Emokpae, M., & Mrakpor, B. (2016). Do Sex Differences in Respiratory Burst Enzyme Activities Exist in Human Immunodeficiency Virus-1 Infection? *Medical Sciences*, *4*(4), 19. https://doi.org/10.3390/medsci4040019

Er-Lukowiak, M., Hänzelmann, S., Rothe, M., Moamenpour, D. T., Hausmann, F., Khatri, R., Hansen, C., Boldt, J., Bärreiter, V. A., Honecker, B., Bea, A., Groneberg, M., Fehling, H., Marggraff, C., Cadar, D., Bonn, S., Sellau, J., & Lotter, H. (2023). Testosterone affects type I/type II interferon response of neutrophils during hepatic amebiasis. *Frontiers in Immunology*, *14*. https://doi.org/10.3389/fimmu.2023.1279245

Fardisi, M., Thelen, K., Groenendal, A., Rajput, M., Sebastian, K., Contreras, G. A., & Moeser, A. J. (2023). Early weaning and biological sex shape long-term immune and metabolic responses in pigs. *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-023-42553-9

Forsyth, K. S., Jiwrajka, N., Lovell, C. D., Toothacre, N. E., & Anguera, M. C. (2024). The conneXion between sex and immune responses. In *Nature Reviews Immunology* (Vol. 24, Issue 7, pp. 487–502). Nature Research. https://doi.org/10.1038/s41577-024-00996-9

Ghosh, M. K., Chen, K. hui E., Dill-Garlow, R., Ma, L. J., Yonezawa, T., Itoh, Y., Rivera, L., Radecki, K. C., Wu, Q. P., Arnold, A. P., Muller, H. K., & Walker, A. M. (2021). Sex Differences in the Immune System Become Evident in the Perinatal Period in the Four Core Genotypes Mouse. *Frontiers in Endocrinology*, *12*. https://doi.org/10.3389/fendo.2021.582614

Grieshaber-Bouyer, R., Radtke, F. A., Cunin, P., Stifano, G., Levescot, A., Vijaykumar, B., Nelson-Maney, N., Blaustein, R. B., Monach, P. A., Nigrovic, P. A., Aguilar, O., Allan, R., Astarita, J., Austen, K. F., Barrett, N., Baysoy, A., Benoist, C., Brown, B. D., Buechler, M., … Yoshida, H. (2021). The neutrotime transcriptional signature defines a single continuum of neutrophils across biological compartments. *Nature Communications*, *12*(1). https://doi.org/10.1038/s41467-021-22973-9

Guo, R., Xie, X., Ren, Q., & Liew, P. X. (2024). New insights on extramedullary granulopoiesis and neutrophil heterogeneity in the spleen and its importance in disease. *Journal of Leukocyte Biology*. https://doi.org/10.1093/jleuko/qiae220

Gupta, S., Nakabo, S., Blanco, L. P., O'neil, L. J., Wigerblad, G., Goel, R. R., Mistry, P., Jiang, K., Carmona-Rivera, C., Chan, D. W., Wang, X., Pedersen, H. L., Gadkari, M., Howe, K. N., Naz, F., Dell'orso, S., Hasni, S. A., Dempsey, C., Buscetta, A., … Kaplan, M. J. (2020). *Sex differences in neutrophil biology modulate response to type I interferons and immunometabolism*. https://doi.org/10.1073/pnas.2003603117/-/DCSupplemental

Hann, J., Bueb, J. L., Tolle, F., & Bréchard, S. (2020). Calcium signaling and regulation of neutrophil functions: Still a long way to go. In *Journal of Leukocyte Biology* (Vol. 107, Issue 2, pp. 285–297). John Wiley and Sons Inc. https://doi.org/10.1002/JLB.3RU0719-241R

Haynes, M. E., Sullivan, D. P., & Muller, W. A. (2024). Neutrophil Infiltration and Function in the Pathogenesis of Inflammatory Airspace Disease. In *American Journal of Pathology* (Vol. 194, Issue 5, pp. 628–636). Elsevier Inc. https://doi.org/10.1016/j.ajpath.2023.12.008

Hidalgo, A., Chilvers, E. R., Summers, C., & Koenderman, L. (2019). The Neutrophil Life

Cycle. In *Trends in Immunology* (Vol. 40, Issue 7, pp. 584–597). Elsevier Ltd. https://doi.org/10.1016/j.it.2019.04.013

Hofer, M. D., Cheng, E. Y., Bury, M. I., Xu, W., Hong, S. J., Kaplan, W. E., & Sharma, A. K. (2015). Androgen supplementation in rats increases the inflammatory response and prolongs urethral healing. *Urology*, *85*(3), 691–697. https://doi.org/10.1016/j.urology.2014.11.025

Hreha, T. N., Collins, C. A., Cole, E. B., Jin, R. J., & Hunstad, D. A. (2024). Androgen exposure impairs neutrophil maturation and function within the infected kidney. *MBio*, *15*(2). https://doi.org/10.1128/mbio.03170-23

Kabutomori, O., Yanagihara, T., Iwatani, Y., Kawarazaki, A., & Kabutomori, M. (1999). Sex difference in myeloperoxidase activity of neutrophils [3]. In *American Journal of Hematology* (Vol. 60, Issue 4, pp. 312–313). https://doi.org/10.1002/(SICI)1096-8652(199904)60:4<312::AID-AJH13>3.0.CO;2-K

Kay, E., Gomez-Garcia, L., Woodfin, A., Scotland, R. S., & Whiteford, J. R. (2015). Sexual dimorphisms in leukocyte trafficking in a mouse peritonitis model. *Journal of Leukocyte Biology*, *98*(5), 805–817. https://doi.org/10.1189/jlb.3a1214-601rr

Klein, S. L., & Flanagan, K. L. (2016). Sex differences in immune responses. In *Nature Reviews Immunology* (Vol. 16, Issue 10, pp. 626–638). Nature Publishing Group. https://doi.org/10.1038/nri.2016.90

Lotter, H., Jacobs, T., Gaworski, I., & Tannich, E. (2006). Sexual dimorphism in the control of amebic liver abscess in a mouse model of disease. *Infection and Immunity*, *74*(1), 118–124. https://doi.org/10.1128/IAI.74.1.118-124.2006

Mackey, E., Thelen, K. M., Bali, V., Fardisi, M., Trowbridge, M., Jordan, C. L., & Moeser, A. J. (2020). *Perinatal androgens organize sex differences in mast cells and attenuate anaphylaxis severity into adulthood*. https://doi.org/10.1073/pnas.1915075117/-/DCSupplemental

Madalli, S., Beyrau, M., Whiteford, J., Duchene, J., Nandhra, I. S., Patel, N. S. A., Motwani, M. P., Gilroy, D. W., Thiemermann, C., Nourshargh, S., & Scotland, R. S. (2015). Sex-specific regulation of chemokine Cxcl5/6 controls neutrophil recruitment and tissue injury in acute inflammatory states. *Biology of Sex Differences*, *6*(1). https://doi.org/10.1186/s13293-015-0047-5

Marciano, B. E., Spalding, C., Fitzgerald, A., Mann, D., Brown, T., Osgood, S., Yockey, L., Darnell, D. N., Barnhart, L., Daub, J., Boris, L., Rump, A. P., Anderson, V. L., Haney, C., Kuhns, D. B., Rosenzweig, S. D., Kelly, C., Zelazny, A., Mason, T., … Holland, S. M. (2015). Common severe infections in chronic granulomatous disease. *Clinical Infectious Diseases*, *60*(8), 1176–1183. https://doi.org/10.1093/cid/ciu1154

Mayadas, T. N., Cullere, X., & Lowell, C. A. (2014). The multifaceted functions of neutrophils. *Annual Review of Pathology: Mechanisms of Disease*, *9*, 181–218. https://doi.org/10.1146/annurev-pathol-020712-164023

Menees, K. B., Earls, R. H., Chung, J., Jernigan, J., Filipov, N. M., Carpenter, J. M., & Lee, J. K. (2021). Sex- and age-dependent alterations of splenic immune cell profile and NK cell phenotypes and function in C57BL/6J mice. *Immunity and Ageing*, *18*(1). https://doi.org/10.1186/s12979-021-00214-3

Meurens, F., Summerfield, A., Nauwynck, H., Saif, L., & Gerdts, V. (2012). The pig: A model for human infectious diseases. In *Trends in Microbiology* (Vol. 20, Issue 1, pp. 50–57). https://doi.org/10.1016/j.tim.2011.11.002

Molloy, E. J., O'Neill, A. J., Grantham, J. J., Sheridan-Pereira, M., Fitzpatrick, J. M., Webb, D. W., & Watson, R. W. G. (2003). Sex-specific alterations in neutrophil apoptosis: The role of estradiol and progesterone. *Blood*, *102*(7), 2653–2659. https://doi.org/10.1182/blood-2003-02-0649

Nadkarni, S., Cooper, D., Brancaleone, V., Bena, S., & Perretti, M. (2011). Activation of the annexin A1 pathway underlies the protective effects exerted by estrogen in polymorphonuclear leukocytes. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(11), 2749–2759. https://doi.org/10.1161/ATVBAHA.111.235176

Ng LG, Ostuni R, Hidalgo A. (2019) Heterogeneity of neutrophils. *Nat Rev Immunol*, 19(4):255-265. https://doi.org/10.1038/s41577-019-0141-8

Osgood, R. S., Kasahara, D. I., Tashiro, H., Cho, Y., & Shore, S. A. (2019). Androgens augment pulmonary responses to ozone in mice. *Physiological Reports*, *7*(18). https://doi.org/10.14814/phy2.14214

Chonchol, M. (2006.). HEMATOLOGY: ISSUES IN THE DIALYSIS PATIENT Neutrophil Dysfunction and Infection Risk in End-Stage Renal Disease. In *Seminars in Dialysis* 19(4):291-6. https://doi.org/10.1111/j.1525-139X.2006.00175.x.

Ramos-Casals, M., Brito-Zerón, P., Kostov, B., Sisó-Almirall, A., Bosch, X., Buss, D., Trilla, A., Stone, J. H., Khamashta, M. A., & Shoenfeld, Y. (2015). Google-driven search for big data in autoimmune geoepidemiology: Analysis of 394,827 patients with systemic autoimmune diseases. In *Autoimmunity Reviews* (Vol. 14, Issue 8, pp. 670–679). Elsevier B.V. https://doi.org/10.1016/j.autrev.2015.03.008

Rettew, J. A., Huet, Y. M., & Marriott, I. (2009). Estrogens augment cell surface TLR4 expression on murine macrophages and regulate sepsis susceptibility in vivo. *Endocrinology*, *150*(8), 3877–3884. https://doi.org/10.1210/en.2009-0098

Rice, C. M., Davies, L. C., Subleski, J. J., Maio, N., Gonzalez-Cotto, M., Andrews, C., Patel, N. L., Palmieri, E. M., Weiss, J. M., Lee, J. M., Annunziata, C. M., Rouault, T. A., Durum, S. K., & McVicar, D. W. (2018). Tumour-elicited neutrophils engage mitochondrial metabolism to circumvent nutrient limitations and maintain immune suppression. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-07505-2

Rodenas, M. C., Tamassia, N., Cabas, I., Calzetti, F., Meseguer, J., Cassatella, M. A., García-Ayala, A., & Mulero, V. (2017). G Protein-Coupled Estrogen Receptor 1 Regulates Human Neutrophil Functions. *Biomedicine Hub*, *2*(1), 1–13. https://doi.org/10.1159/000454981

Roos, D., van Leeuwen, K., Madkaikar, M., Kambli, P. M., Gupta, M., Mathews, V., Rawat, A., Kuhns, D. B., Holland, S. M., de Boer, M., Kanegane, H., Parvaneh, N., Lorenz, M., Schwarz, K., Klein, C., Sherkat, R., Jafari, M., Wolach, B., den Dunnen, J. T., Köker, M. Y. (2023). Hematologically important mutations: Leukocyte adhesion deficiency (second update). *Blood Cells, Molecules, and Diseases*, *99*. https://doi.org/10.1016/j.bcmd.2023.102726

Scalerandi, M. V., Peinetti, N., Leimgruber, C., Rubio, M. M. C., Nicola, J. P., Menezes, G. B., Maldonado, C. A., & Quintar, A. A. (2018). Inefficient N2-like neutrophils are promoted by androgens during infection. *Frontiers in Immunology*, *9*(SEP). https://doi.org/10.3389/fimmu.2018.01980

Schmiedel, B. J., Singh, D., Madrigal, A., Valdovino-Gonzalez, A. G., White, B. M., Zapardiel-Gonzalo, J., Ha, B., Altay, G., Greenbaum, J. A., McVicker, G., Seumois, G., Rao, A., Kronenberg, M., Peters, B., & Vijayanand, P. (2018). Impact of Genetic Polymorphisms on Human Immune Cell Gene Expression. *Cell*, *175*(6), 1701-1715.e16. https://doi.org/10.1016/j.cell.2018.10.022

Scotland, R. S., Stables, M. J., Madalli, S., Watson, P., & Gilroy, D. W. (2011). Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. *Blood*, *118*(22), 5918–5927. https://doi.org/10.1182/blood-2011-03-340281

Sengupta, S., Caldwell, C. C., & Nomellini, V. (2020). Distinct Neutrophil Populations in the Spleen During PICS. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.00804

Soehnlein, O., Steffens, S., Hidalgo, A., & Weber, C. (2017). Neutrophils as protagonists and targets in chronic inflammation. In *Nature Reviews Immunology* (Vol. 17, Issue 4, pp. 248–261). Nature Publishing Group. https://doi.org/10.1038/nri.2017.10

Tang, J. J., Pan, Y. F., Chen, C., Cui, X. L., Yan, Z. J., Zhou, D. X., Guo, L. N., Cao, D., Yu, L. X., & Wang, H. Y. (2022). Androgens drive sexual dimorphism in liver metastasis by promoting hepatic accumulation of neutrophils. *Cell Reports*, *39*(12). https://doi.org/10.1016/j.celrep.2022.110987

Taslem Mourosi, J., Anwar, S., & Hosen, M. J. (2022). The sex and gender dimensions of COVID-19: A narrative review of the potential underlying factors. In *Infection, Genetics and Evolution* (Vol. 103). Elsevier B.V. https://doi.org/10.1016/j.meegid.2022.105338

VanderWaal, K., & Deen, J. (2018). Global trends in infectious diseases of swine. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(45), 11495–11500. https://doi.org/10.1073/pnas.1806068115

Villar, I. C., Scotland, R. S., Khambata, R. S., Chan, M., Duchene, J., Sampaio, A. L., Perretti, M., Hobbs, A. J., & Ahluwalia, A. (2011). Suppression of endothelial p-selectin expression contributes to reduced cell trafficking in females: An effect independent of no and prostacyclin. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(5), 1075–1083. https://doi.org/10.1161/ATVBAHA.111.223545

Wang, Q., Ma, J., Gong, Y., Zhu, L., Tang, H., Ye, X., Su, G., Huang, F., Tan, S., Zuo, X., Gao, Y., & Yang, P. (2024). Sex-specific circulating unconventional neutrophils determine immunological outcome of auto-inflammatory Behçet's uveitis. *Cell Discovery*, *10*(1). https://doi.org/10.1038/s41421-024-00671-2

Zhang, P., Fu, Y., Ju, J., Wan, D., Su, H., Wang, Z., Rui, H., Jin, Q., Le, Y., & Hou, R. (2019). Estradiol inhibits fMLP-induced neutrophil migration and superoxide production by upregulating MKP-2 and dephosphorylating ERK. *International Immunopharmacology*, *75*. https://doi.org/10.1016/j.intimp.2019.105787

CHAPTER 2: IMPACT OF EARLY WEANING, BIOLOGICAL SEX, AND CASTRATION ON ILEAL MUCOSAL TRANSCRIPTOMIC RESPONSES TO LPS CHALLENGE IN PREPUBERTAL PIGS

2.1. ABSTRACT

Early-life adversity (ELA) and biological sex are important factors that influence developmental trajectories and disease risk. ELA is linked to the development of a hyperactive immune phenotype, which increases the risk of chronic inflammatory diseases later in life. Females are more susceptible to the adverse effects of ELA; however, the specific mechanisms behind this increased vulnerability are still not fully understood. This study aimed to explore the impact of early weaning stress and sex on mucosal immune responses in pigs. Female pigs, intact male pigs, and castrated male pigs were weaned at either 15 or 28 days of age. At 70 days of age, these animals received an intramuscular injection of 25 μg/kg of lipopolysaccharide (LPS) to induce inflammation or saline. Ileum mucosa samples were collected 4 hours after the challenge for RNA sequencing. LPS exposure was associated with increased expression of genes involved in stress pathways, such as *HSPA8*, *HSP90AB1*, *STIP1*, *FKBP4*, and *PTGES3.* This response was common to all groups and represented a conserved response to LPS. In female pigs, LPS inhibited the expression of genes involved in cell cycle progression independent of wean age and higher expression of genes involved in extracellular matrix dynamics in the early-wean group. In late-weaned male and castrated male pigs, there was higher expression of genes involved in RNA metabolism and transcription regulation, and early-weaning stress increased the enrichment of cytokine signaling gene sets, especially for the male group, where *IFRD1*, *ATF4*, and *CEBPB* were amount the most significantly induced genes. Castrated male pigs showed a more conserved immune response across wean age and had higher expression of genes involved in protein synthesis. Notably, *PLA2G2A*, a known regulator of intestinal epithelial differentiation and mucosal immunity, was the most differentially expressed gene in late-weaned males but not in

castrated or early-weaned groups. Overall, this study highlights both conserved and sex-specific transcriptomic responses to LPS in prepubertal pigs, demonstrating that early-weaning stress affects later-life mucosal immune responses. These findings may inform future studies with more targeted, mechanistic approaches and contribute to developing strategies that mitigate the long-term effects of ELA on intestinal health in both humans and animals.

2.2. INTRODUCTION

The perinatal period is a developmental window during which environmental cues are sensed; these stimuli can alter epigenetic programming and have lasting effects. Adverse experiences during this sensitive period, also termed early-life adversities (ELA), such as malnutrition, infections, parental absence, or exposure to violence, can disrupt developmental trajectories and result in greater risk of mental health problems and a variety of chronic inflammatory diseases, including inflammatory bowel disease (Agrawal et al., 2021; Merrick et al., 2019). Although the exact mechanisms underlying these associations are still not well understood, individuals exposed to ELA have heightened inflammatory responses throughout life and elevated serum levels of proinflammatory markers, including C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) (Baumeister et al., 2016; Kuhlman et al., 2020).

Biological sex is another factor that influences immune function and disease susceptibility. While males are generally more vulnerable to infectious diseases and have a higher mortality rate from diseases such as COVID-19 (Mourosi et al., 2022), females tend to mount more effective immune responses, which aids in pathogen clearance but also predisposes them to heightened inflammatory reactions and autoimmune diseases (Forsyth et al., 2024; Ramos-Casals et al., 2015). Moreover, females are more susceptible than males to the effects of ELA (Baldwin et al., 2018;

Kirsch et al., 2024). Sex steroids and chromosome complement influence these differences. In adults, estrogens generally enhance immune responses, while androgens are immunosuppressive (Dunn et al., 2024; Rettew et al., 2008, 2009). Additionally, the X chromosome encodes multiple immune-related genes, some of which escape inactivation, further amplifying immune activity in females (Forsyth et al., 2024). Importantly, studies suggest that sex differences in immune function emerge early in life and are driven by perinatal androgen surges, which play a crucial role in male sexual differentiation and immune programming (Mackey et al., 2020).

Despite growing recognition of the impact of ELA and sex differences on immune function, the cellular and molecular mechanisms linking biological sex and early-life stress to immune programming and long-term health outcomes remain poorly understood. Animal models can provide valuable insights into these mechanisms, and the pig (*Sus scrofa*) is increasingly recognized as a relevant preclinical model due to its genomic, anatomical, physiological, and immunological similarities to humans (Schaaf & Gonzalez, 2022; Meurens et al., 2012). Additionally, advancing our understanding of these mechanisms in pigs is particularly important given their economic significance and role in global food security.

In commercial pig farms, weaning typically occurs abruptly between 2-4 weeks, contrasting with the gradual weaning process in more natural environments, which is usually completed when the animal reaches 10-12 weeks of age (Moeser et al., 2017). This early, sudden weaning exposes piglets to the cumulative stress associated with maternal separation, transportation, dietary changes, mixing with unfamiliar piglets, reestablishing hierarchy, and vaccinations. These stressors coincide with a critical period for the pig's immune and gastrointestinal system development, leading to later-life issues, such as chronic diarrhea linked to increased mast cell activity and hypersensitivity of enteric cholinergic neurons (Medland et al.,

2016; Pohl et al., 2017).

Thus, early weaning stress in pigs is a valid translational model for studying the effects of ELA on intestinal development and mucosal integrity (Medland et al., 2016; Moeser et al., 2017). And similar to humans, female pigs demonstrate greater susceptibility to these ELA-induced effects than males (Pohl et al., 2017; Medland et al., 2016). These sex differences were also evident in immunological and metabolic parameters, with early-weaned female pigs showing greater neutrophil-to-lymphocyte ratio and lower glucose utilization than males when challenged with lipopolysaccharide (LPS) in a model of systemic immune activation (Fardisi et al., 2023).

Thus, in this study, we aimed to investigate how early weaning stress, biological sex, and castration status influence gene expression in the ileal mucosa following LPS-induced immune activation. We will achieve this by analyzing the ileal mucosal transcriptome of late- and early-weaned female, male, and castrated male pigs. We hypothesized that early weaning stress will enhance the expression of immune genes and that female, male, and castrated male pigs will have distinct transcriptomic profiles.

2.3. METHODS

2.3.1. Animals

The University Institutional Animal Care and Use Committee (IACUC) approved and supervised all animal procedures under Protocol PROTO201900090.

This study utilized 71 pigs (Yorkshire x Duroc cross, *Sus scrofa*), comprising twenty-three females and forty-eight males, housed at the Michigan State University Swine Research and Teaching Center in East Lansing, MI, USA. During the first week of life, the piglets were selected from a group of twenty-six sows (parity 3 to 5) in 5 cohorts. Twenty-four of the males were castrated within the first ten days after birth. Within each sow, piglets were matched by sex into

early-weaned (EW, weaned at 15 days) and late-weaned (LW, weaned at 28 days) littermate pairs to reduced reduce the impact of variability of the sow to wean age and sex comparisons. At 70 days, pigs were randomly assigned to one of two treatment groups: saline control or LPS challenge (25 μg/kg LPS, E. coli O55:B5, Sigma, Catalog # L2880), as described by Fardisi et al. (2023). This design resulted in 12 experimental groups with six animals per group, except for the early-weaned female group treated with LPS, which included five animals.

2.3.2. RNA Extraction

Ileal mucosa samples were used for bulk RNA sequencing. RNA was extracted from frozen tissue samples using TRIzol reagent (Thermo Fisher Scientific). To ensure complete cell lysis and release of RNA into the TRIzol solution, the samples were homogenized with 2.3 mm silica beads using a Precellys bead mill homogenizer. After centrifugation, the supernatant containing the RNA was separated from the beads and the cellular debris. Further purification of the RNA was achieved through chloroform separation, which effectively separated the aqueous phase containing RNA from other cellular components. The total RNA extraction was then performed using the RNeasy Mini Kit (QIAGEN), following the manufacturer's protocol.

2.3.3. Sequencing

The Van Andel Genomics Core performed the sequencing. Libraries were prepared from 500 ng of total RNA using the KAPA mRNA Hyperprep kit (v8.23) (Kapa Biosystems, Wilmington, MA USA). The RNA was fragmented to 300-400 bp. Before PCR amplification, cDNA fragments were ligated to IDT for Illumina TruSeq UD Indexed adapters (Illumina Inc, San Diego CA, USA). The quality and quantity of the finished libraries were assessed using a combination of Fragment Analyzer (Agilent Technologies, Inc), QuantiFluor® dsDNA System (Promega Corp.), and Kapa Illumina Library Quantification qPCR assays (Kapa Biosystems). Individually indexed libraries

were pooled, and 2x50 bp sequencing was performed on an Illumina NovaSeq6000 sequencer using an S4 sequencing kit (Illumina Inc., San Diego, CA, USA) to an average depth of 45M reads per sample. Base calling was performed with Illumina RTA3, and the output of NCS was demultiplexed and converted to FastQ format with Illumina Bcl2fastq2 v2.20.

2.3.4. Bioinformatics

The FastQ files were analyzed at the MSU Bioinformatics Core using the nf-core RNAseq pipeline, version 3.14.0, managed through Nextflow version 23.10.1. Data quality was initially assessed with FASTQC version 0.11.9, followed by adapter trimming using Trimgalore version 0.6.7. Gene expression was quantified with Salmon version 1.9.0, referencing the Scrofa11.1 genome (version GCA_000003025.6, Sus scrofa - Ensembl genome browser 111). The counts matrix was corrected for cohort effect using the SVA R library. Normalization and differential gene expression analysis were performed with DESeq2 R version 1.28.0, applying prefiltering criteria of 10 counts in at least 12 samples. 16,573 genes met these criteria and were considered for further analysis. All genes with a p-value ≤ 0.05 were considered as significantly differentially expressed.

The normalized counts were the input for the enrichment analysis performed in Gene Set Enrichment Analysis (GSEA version 4.3.3). The Reactome pathways, available through g:Profiler (version *e111_eg58_p18_f463989d*, 25/01/2024 update) for the organism *Sus scrofa,* were used as the reference gene sets. GSEA was performed using gene set permutation and 149 as seed for permutation. In addition, we selected only pathways with 1 to 500 genes. All other parameters were set to default values. All pathways with a false discovery rate ≤ 0.05 were considered significant.

We used the Enrichment Map application in Cytoscape (version 3.10.2) to visualize and compare the GSEA results from the different groups. The enrichment results for female, male, and

castrated male groups were uploaded concomitantly to facilitate direct comparisons within late and early wean conditions. Pathways were represented as nodes, and their relationships were defined based on shared genes. A false discovery rate (FDR) threshold of 0.05 was applied to filter significant pathways and an edge similarity cutoff of 0.375 was used to determine pathway connectivity. This approach enabled the identification of clusters of functionally related gene sets, allowing for a more intuitive interpretation and comparison of the enrichment results across groups.

The g:Profiler website was also used for enrichment analysis of the intersecting genes. The Ensembl gene identifiers for *Sus scrofa* were used as input, and an adjusted p-value threshold of ≤ 0.05 (Bonferroni correction) was defined for pathway significance. Reactome and KEGG pathways were considered.

2.4. RESULTS

2.4.1. Principal component and differential gene expression analyses highlight biological sex, castration, and weaning age effects on ileal transcriptomic responses to LPS

Mean sequencing quality scores were 35, and sequencing coverage (Figure S1) ranged from 40× to 50× in most samples, indicating that the data is of high quality, consistent, and suitable for downstream analyses.

To investigate the impact of biological sex, castration status, and wean age, on the ileal mucosa transcriptomic response to LPS-induced immune activation, we performed principal component analysis (PCA). The resulting PCA plot (Figure 1A) revealed that principal component 1 (PC1) accounted for 14.61% of the total variance, while principal component 2 (PC2) accounted for 8.51%. Variance in PC1 was primarily driven by LPS treatment, with the most variable genes including *HSPA4L* (heat shock protein family A member 4-like), *HSPH1* (heat shock protein family

H (Hsp110) member 1), *BAG3* (BAG cochaperone 3), *DNAJA4* (DnaJ heat shock protein family (Hsp40) member A4), *HSPA6* (heat shock protein family A (Hsp70) member 6), and *CRYAB* (crystallin alpha B) (Figure 1B). These genes encode heat shock proteins (HSPs), chaperones that fold newly synthesized or misfolded proteins, a conserved cellular response to stress (Figure 1B).

In PC2, samples were segregated based on biological sex, with females distributed in the upper part of the graph, while male and castrated male pigs were grouped in the lower half. The Y-chromosome genes such as *DDX3Y* (DEAD-box helicase 3 Y-linked), *ZFY* (zinc finger protein Y-linked), *EIF2S3* (eukaryotic translation initiation factor 2 subunit gamma), *USP9Y* (Ubiquitin specific peptidase 9 Y-linked), and *KDM5D* (lysine demethylase 5D), contributed most to variance in this component. These genes are expressed in male and castrated male pigs, not females (Figure 1C).

Differential gene expression analysis was conducted to explore the effects of LPS treatment on gene expression. Table 2.1 shows the number of differentially expressed genes (DEGs) between LPS and saline-treated animals in each group. Male pigs had more DEGs than females at both late wean (3,448 vs. 2,217 DEGs) and early wean (3,613 vs. 2,080 DEGs), highlighting consistent sex differences in the mucosal immune response, irrespective of the effect of age at weaning. Notably, late-weaned castrated male pigs exhibited the highest number DEGs in response to LPS challenge 4,569, surpassing the number of DEGs of both female and male pigs. Moreover, early weaning stress was associated with a 55% (2,476) decrease in LPS-induced DEGs, which could indicate an interaction between age at weaning and castration on mucosal immune development.

2.4.2. Conserved Transcriptomic Response to LPS Across Sex and Weaning Age

Two hundred twenty-four genes were differentially expressed and similarly regulated in all groups. These genes could represent a conserved response to LPS exposure across age at weaning

and sexes. They provide us with a reference point to compare group-specific responses. One hundred sixty-four genes showed higher expression in LPS-treated samples, while sixty genes had lower expression levels, as illustrated in Figure 2A. Moreover, using the g:Profiler website, we performed functional enrichment to further explore these markers. The genes with greater abundance in LPS-treated samples were predominantly associated with stress response and were significantly enriched in gene sets such as *cellular response to heat stress* (11 genes), the *HSP90 chaperone cycle for steroid hormone receptors (SHR) in the presence of ligand* (9 genes), and *HSF1-dependent transactivation* (7 genes) (Figure 2B).

Key genes contributing to this enrichment were *HSPA8* (Heat Shock Protein Family A (Hsp70) Member 8), *HSP90AB1* (Heat Shock Protein 90 Alpha Family Class B Member 1), *STIP1* (Stress-Induced Phosphoprotein 1), *FKBP4* (FKBP Prolyl Isomerase 4), and *PTGES3* (Prostaglandin E Synthase 3). These genes are of interest because they are known to interact with nuclear steroid receptors (Baker et al., 2019), which play a central role in the long-term effects of early life adversity in the gut and sex differences in the immune response. Furthermore, these genes showed consistently larger $\log_2$ fold changes and statistical significance in male and castrated males than in female pigs (Table 2.2). In Figure 2C, the STRING network shows these genes form a dense interacting cluster, reflecting their tendency to assemble into large functional protein complexes.

2.4.3. The Most Significant Changes in Gene Expression After LPS-Induced Immune Activation

In addition to investigating the common aspects of the response to LPS-induced immune activation across biological sex, castration status, and wean groups, our objective was to understand the differences in gene expression after LPS treatment. To achieve this, we analyzed

each group's transcriptomic response to LPS, first investigating the 20 most significant DEGs. Then, we conducted a GSEA for each group to better understand the gene classes and possible biological functions that were most significantly affected by LPS treatment.

2.4.3.1. Inhibition of the Cell Cycle is a Conserved Response to LPS in Female Pigs, While Early Weaning Stress is Associated with High Differential Expression of Extracellular Matrix Genes

In late-weaned female pigs, *PLA2G2A* (phospholipase A2 group IIA), a gene from chromosome 6, exhibited the most significant increase in expression in response to LPS, with a $\log_2$ fold change of 24.6 and an adjusted p-value of $5.61e^{-12}$ (Figure 3A). This phospholipase is a negative regulator of intestinal stem cell proliferation and differentiation and is also involved in prostaglandin-E2 synthesis, a key mediator of intestinal inflammation (Schewe et al., 2016). In addition, the heat shock protein genes (*HSPA1B, HSPH1, HSPA4L,* and *HSPB1*) represented the most abundant class among the top 20 most significantly differentially expressed genes, emphasizing their central role in the LPS-induced stress response.

Supporting these findings, gene sets associated with the cell cycle, including the *S phase* and *DNA synthesis*, were among the most significantly negatively enriched pathways. In contrast, gene sets such as the *HSP90 chaperone cycle for steroid hormone receptors (SHR) in the presence of ligand* and *HSF1-dependent transactivation* were among the most significantly positively enriched, further highlighting the activation of stress response pathways following LPS exposure (Figure 3C).

In contrast, early-weaned female pigs exhibited increased mRNA levels of genes involved in collagen synthesis and maturation, including *SERPINH1* (serpin family H member 1), *LOXL2* (lysyl-oxidase-like 2), and *COL4A5* (collagen type IV alpha-5 chain) (Figure 3A). Additionally,

THBS1 (thrombospondin 1) and *WFIKKN1* (WAP, Kazal, immunoglobulin, Kunitz, and NTR domain-containing protein 1), which regulate the activity of transforming growth factor-beta (TGF-β), a key driver of extracellular matrix gene expression, also exhibited increased expression. This pattern was further emphasized by GSEA, which identified positive enrichment of genes involved in *extracellular matrix organization*, *degradation of the extracellular matrix*, and *collagen formation* pathways (Figure 3B).

Markers of immune cell adhesion and activation, such as *MADCAM1* (mucosal vascular addressin cell adhesion molecule 1), *CD44*, and integrins (*ITGA1*, *ITGA9*, *ITGB8*, and *ITGB7*), as well as tissue remodeling markers, including multiple collagen genes (*COL4A6, COL18A1, COL5A1, COL4A2, COL5A2, and COL12A1*), metalloproteinases (*MMP2, MMP14, ADAMTS2*, and *ADAMTS5*), and enzymes involved in collagen syntheses, such as lysyl hydroxylases (*PLOD1* and *PLOD3*) and lysyl oxidases (*LOXL4, LOXL2*, and *LOX*) were at among the most differently expressed genes within the positively enriched gene sets in early-weaned female pigs (Figure 3G).

Moreover, similar to the late-weaned group, early-weaned female pigs exhibited strong negative enrichment for gene sets involved in the cell cycle, including *mitotic metaphase and anaphase*, *S phase*, and *DNA synthesis*. In both weaning groups, proteasome subunits (PSMs) represented the most abundant type of genes enriched within these pathways (Figure 3E-F). These findings suggest that LPS suppresses cell cycle and proteasome activity in the ileum mucosa of females, a pattern not affected by weaning age.

2.4.3.2. RNA Metabolism and Inflammation Gene Expression Changes in LPS-Treated Male Pigs at Late Wean and Early Wean Conditions.

Similarly to what was observed in the late-weaned female group, *PLA2G2A* exhibited the most significant increase in expression following LPS exposure in late-weaned male pigs, with a

log_2 fold change of 23.1 and an adjusted p-value of $3.19e^{-10}$. In addition, genes encoding molecular chaperones also represented the most abundant class among the top 20 most significant DEGs, including *HSPH1, HSPA1B, HSPB1, HSPD1, FKBP4, STIP1, BAG3*, and *CRYAB*, reinforcing their central role in the response to LPS (Figure 4A).

Interestingly, the most significantly enriched gene sets in the late-weaned male group were related to RNA metabolism (Figure 4C). This enrichment was driven by genes involved in mRNA degradation, including *ZFP36L1* (ZFP36 ring finger protein-like 1), *UPF1* (UPF1 RNA helicase and ATPase), and *SMG7* (SMG7 nonsense-mediated mRNA decay factor). And genes associated with splicing regulation, such as *PRPF3* (PRPF3 pre-mRNA processing factor 3), *RBM10* (RNA binding motif protein 10), and *RBM22* (RNA binding motif protein 22) (Figure 4E). These findings highlight the significant involvement of post-transcriptional regulatory mechanisms in the mucosa immune response of late-weaned male pigs to LPS exposure.

Conversely, early-weaned male pigs exhibited increased expression of inflammatory markers following LPS exposure. The top 20 most significantly differentially expressed genes included transcription factors that regulate pro-inflammatory cytokine production, such as *IFRD1* (interferon-related developmental regulator 1) and *CEBPB* (CCAAT enhancer binding protein beta). Genes encoding proteins involved in cytokine signaling amplification were also significantly elevated, such as *RIPK2* (receptor-interacting serine/threonine kinase 2), *OSMR* (oncostatin M receptor), and *GPR4* (G protein-coupled receptor 4) (Figure 4B).

These findings were further corroborated by GSEA (Figure 4D), which revealed that LPS treatment was linked to the positive enrichment of cytokine signaling pathways, including *cytokine signaling in immune system*, *interferon signaling,* and *signaling by interleukins*. Notably, key mediators such as signal transducers and activators of transcription (*STAT2, STAT3, STAT4*) and

the transcription factor p65 (*RELA*) were positively enriched (Figure 4F). These factors are essential regulators of pro-inflammatory cytokine production and reinforce the strong activation of inflammatory networks in early-weaned male pigs in response to LPS exposure.

In late-weaned male pigs, gene sets associated with energy metabolism were negatively enriched (Figure 4C), suggesting suppression of energy metabolism following LPS exposure. Moreover, early-weaned males exhibited negative enrichment of DNA repair-related gene sets, including *resolution of abasic sites (AP sites)*, *PCNA-dependent long patch base excision repair*, and *lagging strand synthesis*.

2.4.3.3. Castrated Male Pigs Exhibit Increased Expression of Protein Synthesis Genes Following LPS Exposure

Unlike female and intact male pigs, the top genes and enriched gene sets were relatively consistent across wean groups in castrated males. Chaperone-encoding genes, including *HSPH1, HSPA1B, HSPA8, HSPB1, BAG3*, and the cochaperone *STIP1*, were consistently among the top 20 most significantly DEGs in both late- and early-weaned castrated males (Figure 5A-B). Additionally, further underscoring the conserved role of chaperones in the LPS response of castrated males, *HSP90AB1, HSPD1, FKBP4, DNAJA1,* and *DNAJB4* were among the top genes in the late-weaned group, and *DNAJA4* was among the most significant genes in the early-weaned group.

Accordingly, the top five positively enriched gene sets in late- and early-weaned castrated males were associated with protein synthesis and translation regulation (Figure 5C-D). These pathways included genes encoding ribosomal protein subunits, such as ribosomal proteins small subunit (RPSs) and ribosomal protein large subunit (RPLs), as well as eukaryotic initiation factors (EIFs), which were abundant and contributed significantly to the enrichment of these gene sets

(Figure 5E-F). Additionally, the increased number of DEGs observed in the late-wean group (Table 1) suggests that castrated male-specific response to LPS is amplified in late-wean conditions.

Pathway analysis of the 3,348 genes differentially expressed in the late-weaned but not the early-weaned castrated male group further revealed significant enrichment in pathways related to ribosome and translation (Figure S2A-B). These findings, combined with the consistently higher expression of chaperone genes and genes encoding ribosomal proteins and eukaryotic initiation factors across both weaning groups, underscore the central role of translational machinery in the immune response of castrated males.

2.4.4. Differences in Ileal Mucosa Gene Expression Associated with Biological Sex, Castration Status, and Early Weaning Stress

The analysis of the top enriched gene sets highlights the genes most strongly associated with the immune response of each experimental group. However, focusing on only the most enriched gene sets, we may overlook additional sets of genes that are either uniquely differentially expressed in specific groups or shared across groups but with lower levels of enrichment. A broader analysis of all significantly enriched gene sets (adjusted p-value ≤ 0.05) can help identify shared and distinct biological processes across experimental conditions. To explore these relationships more comprehensively, we used the Enrichment Map application in Cytoscape, which allows us to visualize gene sets enriched in each group and how these gene sets are related to one another based on shared gene content.

The number of gene sets uniquely enriched in late-weaned females, males, and castrated males was 62, 36, and 43, respectively. Additionally, the number of overlapping shared gene sets was limited: two gene sets were shared between females and males, 11 were shared between males and castrated males, no gene sets were commonly enriched between late-weaned female and

castrated male pigs, and a total of five gene sets were shared among all groups (Figure 6A).

In contrast, the number of enriched gene sets is markedly greater in early-weaned animals, especially for females and castrated males. 286 gene sets were uniquely enriched in early-weaned female pigs, 30 in males, and 86 in castrated male pigs. The number of shared gene sets remained low: 22 were shared between females and males, 12 between males and castrated males, and two between females and castrated males. Additionally, only three gene sets were commonly enriched across all groups (Figure 6B).

The limited overlap of gene sets across sex and castration stratus in both wean conditions indicates that female, male, and castrated male pigs engage distinct mechanisms in their response to LPS. Moreover, the higher number of significantly enriched gene sets observed in early-weaned female and castrated male pigs indicates that the stress associated with early weaning primes the ileum mucosa for a more reactive state. These differences reinforce that early-life stress influences long-term mucosal immune programming, with biological sex and castration status further shaping the specific transcriptional landscape (Figure 6C-D).

Among late-weaned animals, gene sets related to cell cycle, DNA repair, immune signaling, and carbohydrate metabolism were negatively enriched in the ileal mucosa of female pigs, but not in males or castrated males. This pattern highlights sex differences in the response to LPS. Notably, early weaning stress exacerbated this characteristic response of females, further amplifying the number of negatively enriched gene sets in these categories. Additionally, early-weaned females exclusively exhibited strong positive enrichment of pathways related to extracellular matrix dynamics, G-protein-coupled receptor (GPCR) signaling, T-cell receptor signaling, and platelet function, suggesting long-term alterations in immune and metabolic programming within the ileum mucosa that may influence their response to infectious challenges later in life (Figure 6C-D).

RNA metabolism, transcription regulation, and cytokine/immune signaling gene sets were positively enriched in late-weaned male and castrated male pigs but not in females, further highlighting sex-specific differences in the mucosal immune response to LPS (Figure 6C).

Interestingly, while there was a large difference in the number of DEGs between late-weaned and early-weaned castrated male groups, the functional classes of enriched gene sets remained consistent, with marked positive enrichment for protein syntheses, transcription regulation, and cytokine signaling in both early and late wean conditions. Male pigs, on the other hand, exhibited a wean-age-dependent shift in functional enrichment despite having a similar number of significant DEGs across weaning conditions. Specifically, late-weaned males showed strong positive enrichment for RNA metabolism gene sets, while early-weaned males shifted toward strong positive enrichment of cytokine signaling and toll-like receptor (TLR) signaling gene sets (Figure 6C-D).

To further assess how weaning age influences gene expression in response to LPS exposure within each sex, we examined the correlation of treatment effects (log2 fold change) for DEGs ($p \leq 0.05$) in either early- or late-weaned pigs (Figure 7A-C). In females, the correlation between log2 fold change values in early- and late-weaned conditions was weak ($r = 0.12$, $p = 1.3e\text{-}12$), suggesting substantial differences in how each gene is regulated depending on weaning age. Interestingly, *PLA2G2A* was one of the genes most differently regulated. In contrast, male and castrated male pigs exhibited a moderate correlation ($r = 0.55$, $p = 2.2e\text{-}16$), indicating a more consistent pattern of gene regulation across weaning ages. These findings suggest that early weaning stress alters gene regulatory dynamics in a more pronounced and divergent manner in females than males and castrated males, potentially reflecting sex-specific differences in responses to early-life stress.

Moreover, considering that comparing groups based on significance using a Venn diagram can result in misleading conclusions due to type II error (Weiner et al., 2022), we assessed how weaning age influences gene expression in response to LPS by examining how genes are regulated between late and early weaning by using a correlation analysis of treatment effects (log2 fold change) within each sex. We are considering only differentially expressed genes (DEGs, $p \leq 0.05$) in early- and late-weaned pigs (Figure 7A-C). In females, the correlation between log2 fold change values across weaning ages was weak ($r = 0.12$, $p = 1.3e\text{-}12$), suggesting substantial differences in gene regulation depending on weaning age. Notably, *PLA2G2A* was among the most differentially regulated genes in response to LPS treatment across weaning groups in female pigs. In contrast, male and castrated male pigs exhibited a moderate correlation ($r = 0.55$, $p = 2.2e\text{-}16$), indicating a more consistent gene regulation pattern across weaning ages. These findings suggest that early weaning stress alters gene regulatory dynamics more profoundly in females than in males and castrated males, potentially reflecting sex-specific differences in responses to early-life stress.

2.5.DISCUSSION

This study investigates how wean age, biological sex, and castration status influence the transcriptome in the ileal mucosa of pigs following LPS stimulation. By identifying conserved and distinct transcriptional responses across these factors, our findings provide novel insights into how early-life stress and sex-related factors shape intestinal immunity and inflammatory regulation.

A key finding was the identification of 224 genes constituting a “core” mucosal transcriptional response to LPS-induced inflammation, conserved across sex and weaning age. Among these, we observed a marked increase in the expression of chaperone-encoding genes, co-chaperones, and other interacting proteins. Of which heat-shock proteins (HSPs) were the most prominent. These chaperones are known to play a dual role during the immune response: they

stabilize newly synthesized and misfolded proteins, preventing cellular damage and apoptosis due to the accumulation of unfolded proteins (Boopathy et al., 2022). They also support the production of new proteins, such as cytokines, required during the immune response. Additionally, HSPs are known to act as damage-associated molecules and to interact with and stabilize signaling molecules downstream of activated toll-like receptor 4 (TLR4), thereby amplifying inflammatory pathways, including NF-κB activation (Ambade et al., 2012).

Importantly, while this core response was conserved across all groups, several chaperone-related, including *HSPA8, HSP90AB1, STIP1, FKBP4,* and *PTGES3*, exhibit stronger differential expression in males and castrated males than in females. These genes encode proteins that directly bind to and interact with nuclear receptors, such as the glucocorticoid and androgen receptors, enhancing their stability and ligand-binding affinity (Baker et al., 2019). Given the role of these receptors in early-weaning stress and the establishment of sex differences early in life, the increased expression of these interacting genes during the mucosal immune response in male pigs, regardless of castration status, suggests greater nuclear receptor activity in the ileal mucosa of male compared to female pigs. Mechanistic studies are needed to determine whether sex differences in nuclear receptor activity in the gut, particularly the glucocorticoid receptor, contribute to sex-specific differences in mucosal immune function in response to early-weaning stress. Notably, the conserved core genes identified here represent promising candidates for the development of therapeutic strategies aimed at improving gut health and immune resilience, independent of sex or early-life conditions.

In addition to the shared transcriptional response, our results reveal striking differences in the mucosal transcriptome among female, male, and castrated male pigs under late and early weaning conditions. Among these, *PLA2G2A* was one of the most significantly differentially

expressed genes following LPS stimulation. It was robustly induced in late-weaned female and male pigs but not in castrated males and the early-weaned groups. Moreover, *PLA2G2A* exhibited one of the most significant disparities in gene expression between late- and early-weaned female groups, further supporting the idea that early-weaning stress alters the ileum mucosal responsiveness to inflammatory stimuli later in life.

PLA2G2A is a marker of mucosal immunity. It is involved in intestinal epithelial stem cell proliferation and differentiation, synthesis of prostaglandin E2, and antimicrobial defense (Shinton et al., 2022; Shin et al., 2019; Schewe et al., 2016). Therefore, altered *PLA2G2A* expression could have direct consequences on the capacity of the gut to repair, regulate inflammation, and defend against pathogens. Additionally, *PLA2G2A* is known to function as an androgen response element in humans (Cistrome Data Browser Toolkit, accessed January 22, 2025), raising the possibility that its expression in pigs may similarly be regulated by androgen receptor signaling, a pathway directly affected by both sex and castration.

In contrast to males and castrated males, LPS-induced inflammation led to a pronounced inhibition of cell cycle progression in the ileal mucosa of female pigs, irrespective of weaning age. This may represent a sex-specific mucosal response, potentially contributing to impaired epithelial renewal during inflammation and the increased susceptibility of females to intestinal barrier dysfunction. Consistent with this, prior studies have linked intestinal inflammation to epithelial cell cycle arrest, impaired epithelial turnover, and increased intestinal permeability (Shi et al., 2019). In addition, sex differences in intestinal epithelial stem cell proliferation have been reported in mice, where females exhibit a higher baseline proliferative capacity in the absence of inflammatory stimuli (Zhou et al., 2018). This suggests that female pigs could rely more on epithelial proliferation for mucosal homeostasis, making them particularly vulnerable to cell cycle

inhibition under inflammatory conditions.

Early-weaning stress was also associated with increased expression of extracellular matrix genes in female pigs following LPS stimulation. Genes involved in collagen syntheses, such as *SERPINH1*, *LOXL2*, and *COL4A5,* were among the most significantly differentially expressed in early-weaned female pigs. Higher expression of these classes of genes is often observed in inflammatory bowel disease cases, a chronic inflammatory condition, where continued inflammatory stimuli contribute to intestinal stiffness and may contribute to reduced nutrient absorption, compromised peristalsis, and, over time, fibrosis (Stewart et al., 2018). Similarly, early weaning stress has been associated with low-grade chronic intestinal inflammation in pigs, with females showing a more pronounced response than castrated males (Pohl et al., 2017). This difference may explain why gene sets associated with the extracellular matrix dynamics are strongly enriched in early-weaned females but not in early-weaned castrated males or late-weaned groups, suggesting that early-weaning stress may sensitize the female gut to adopt a fibrotic-like transcriptional signature upon an inflammatory challenge. It is worth noting that Pohl et al. (2017) did not observe histological changes associated with early weaning stress. Still, the transcriptional changes observed here may represent an early molecular adaptation to inflammation.

Similarly, wean age strongly affects the transcriptional response of the ileal mucosa of male pigs following LPS stimulation. In late-weaned male pigs, gene sets linked to RNA metabolism were strongly positively enriched, including RNA splicing gene sets. Post-transcriptional regulation plays a crucial role in shaping rapid immune responses, as it allows for dynamic gene expression adjustments during different inflammatory response phases. Alternative splicing can increase the complexity of the proteome and can regulate immune responses by generating different protein isoforms with distinct functions (Lai et al., 2021; Janssen et al., 2020). In contrast,

early-weaned males had increased expression of genes related to a more acute inflammatory response. Notably, *IFRD1* and *CEBPB* were two of the most differentially expressed genes in early-weaned males. These transcription factors are central modulators of TLR4-downstream signaling and play critical roles in regulating pro-inflammatory gene expression, including interleukin-6 (IL6) and tumor necrosis factor-alpha (TNF-α) (Ren et al., 2023; Gu et al., 2009). Together, these findings suggest that early-weaning stress primes the male ileal mucosa for a heightened inflammatory response upon LPS challenge, whereas late-weaned males may engage more prominently in post-transcriptional regulatory mechanisms, potentially contributing to a more controlled immune response.

The enrichment of cytokine signaling gene sets in male, but not female pigs, is particularly intriguing, as it appears to contrast with previous studies suggesting that females mount a more robust inflammatory response than males in response to early-weaning stress, including higher TNF-α secretion and higher neutrophil-to-lymphocyte ration (Fardisi et al., 2023). However, an important limitation of our study is that we focused on the response to LPS, relying on log2 fold changes in RNA abundance within each group. This approach does not account for potential baseline differences in gene expression between groups, nor does it consider possible sex-dependent differences in the levels of stored inflammatory mediators, such as mast cell mediators (Pohl et al., 2016).

Despite the notable differences in the number of DEGs between late-weaned (4,568) and early-weaned (2,092) castrated male pigs, the top enriched gene sets were identical, demonstrating a conserved transcriptional response to LPS exposure. The lower number of DEGs in the early-weaned group may be attributed, at least in part, to greater intragroup variability, which could obscure the detection of differentially expressed transcripts. Nonetheless, castrated males across

both weaning groups consistently exhibited increased expression of genes associated with protein synthesis and cytokine signaling following LPS stimulation. This transcriptional pattern may be influenced by the inherently low androgen levels in castrated males or by the additional burden of surgical castration itself, which represents another form of early-life adversity. Notably, surgical castration in pigs, typically performed within the first week of life without analgesia, has been linked to reduced growth rates and increased pre-weaning mortality (Morales et al., 2017), suggesting that this early-life procedure may add to the stress response and shape the transcriptional programming observed in these animals.

Enhanced activity of the translational machinery has been linked to the proliferation and activation of immune cells, such as lymphocytes and dendritic cells (Asmal, 2003; Lelouard et al., 2007). In addition, studies in mice have demonstrated that castration can alter the populations of differentiated mucosal cells, such as goblet cells, enteroendocrine cells, and Paneth cells (Yu et al., 2020) and is associated with an increased number of mucosal immune cells, including innate lymphocytes (Laffont et al., 2017). Taken together, these findings suggest that the observed increase in abundance of mRNA associated with translation in castrated male pigs, but not in intact males or females, may reflect a unique shift in mucosal cell composition and immune cell populations driven by the combined effects of androgen deprivation and castration-induced stress. This altered cellular landscape may underlie the distinct transcriptional response observed in castrated males following LPS exposure.

It is also important to acknowledge that the transcriptional responses observed in this study reflect the ileal mucosa status four hours after a systemic LPS challenge. Therefore, the changes detected may not necessarily result from the direct interaction of LPS with intestinal cells, but rather from the action of systemic inflammatory mediators circulating in response to LPS

exposure. Additionally, group-specific differences in inflammatory mediators, as previously reported (Fardisi et al., 2023), along with potential baseline differences in cytokine receptor expression, may have contributed to the transcriptional patterns observed. These factors highlight the complexity of interpreting mucosal transcriptomic responses following a systemic immune challenge.

In this study, we analyzed the ileal mucosal transcriptome following LPS exposure, uncovering both conserved and sex-specific gene expression patterns in female, male, and castrated male pigs previously weaned at 15 or 28 days. Our findings reveal a conserved role for chaperone-encoding genes in managing cellular stress and immune activation, alongside distinct sex- and wean age-dependent differences in cytokine signaling, extracellular matrix remodeling, cell cycle regulation, and protein synthesis. These results provide novel insights into how early-life stress and sex modulate immune programming in the ileum, potentially contributing to long-term alterations in mucosal function.

By identifying key genes and functional gene sets involved in these different responses, our study provides a foundation for future research integrating transcriptomic and mechanistic approaches to further explore the hyperactive immune phenotype associated with early weaning stress and sex differences in mucosal immune regulation. Given the genetic, anatomical, and physiological similarities between pigs and humans, the pig is a suitable translation model, and our findings offer valuable insights into the mechanisms underlying disease susceptibility in individuals exposed to early-life adversities. Additionally, pigs are a significant livestock species, and this study contributes to the development of strategies for improving swine health and productivity, ultimately supporting more sustainable and resilient livestock production systems.

2.6. TABLES AND FIGURES

Figure 1. Overview of transcriptomic changes in the ileal mucosa of 70-day-old female, male, and castrated pigs weaned at 15 or 28 days following LPS or saline treatment. 1A) PCA was performed using variance-stabilizing transformation from the DESeq2 package, including all 16,573 genes. The size of points represents wean age (small for 15 days, large for 28 days), color represents sex (pink for female, blue for male, green for castrate), and shape indicates treatment (triangle for LPS, circle for saline). 1B-1C) Heatmap of the expression values of the top 30 genes contributing to variability in principal components 1 and 2. The expression values were normalized by row, with red representing the sample with the higher expression values and blue representing the sample with the lower expression. Columns are annotated by wean age, sex, and treatment, while rows indicate their chromosomal location.

Figure 1A

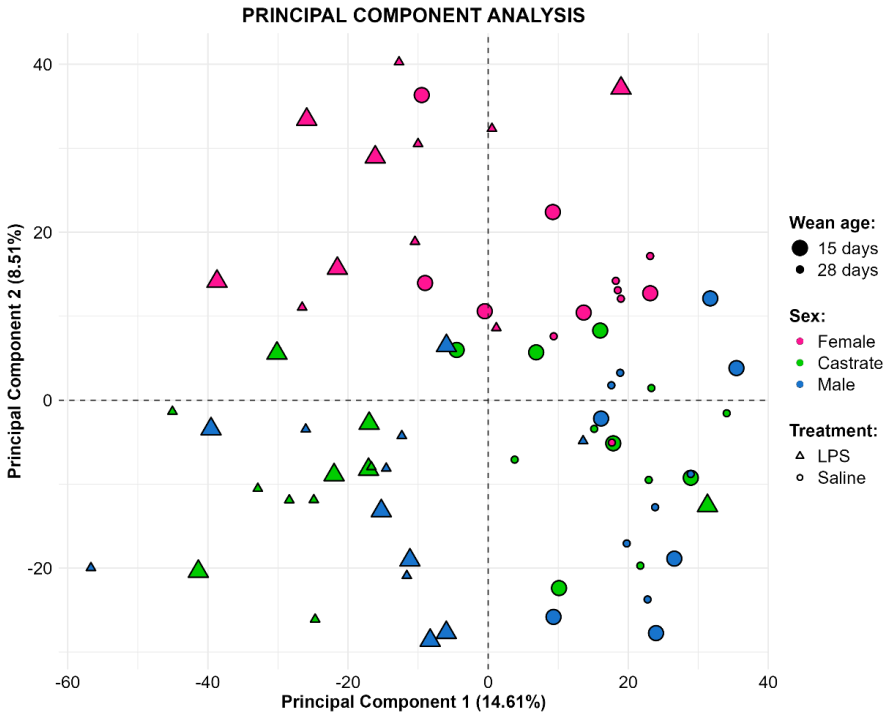

Figure 1 (cont'd).

Figure 1B

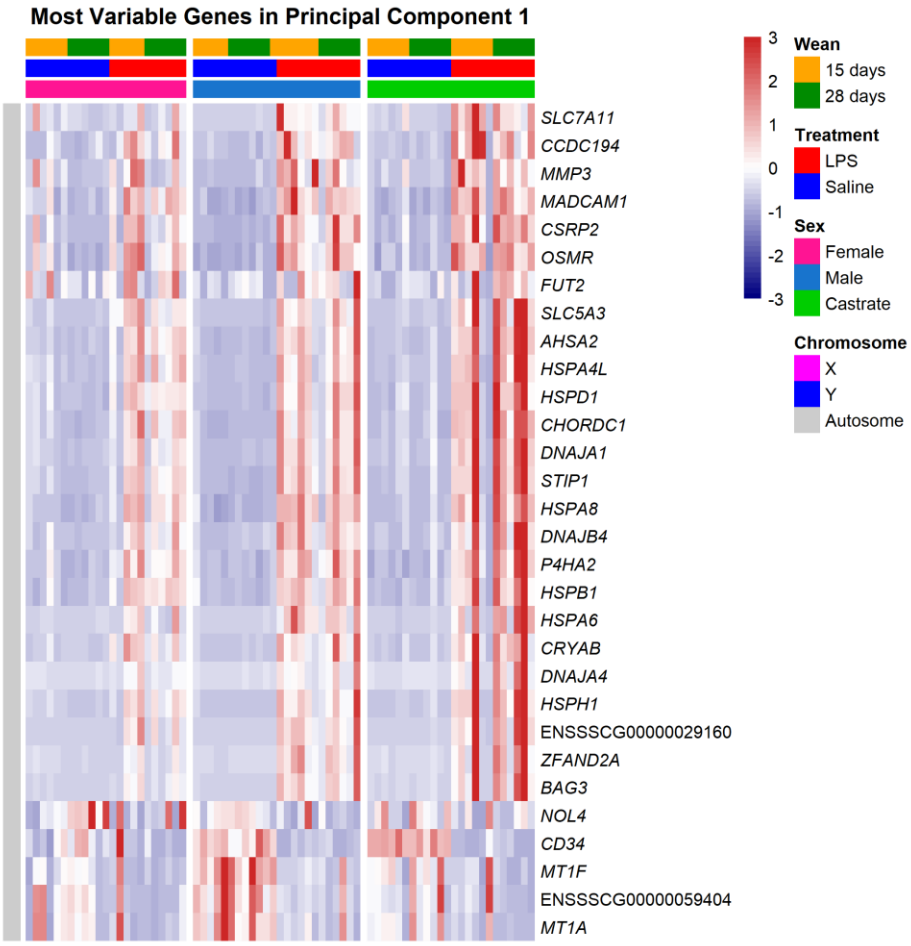

Figure 1 (cont'd)

Figure 1C

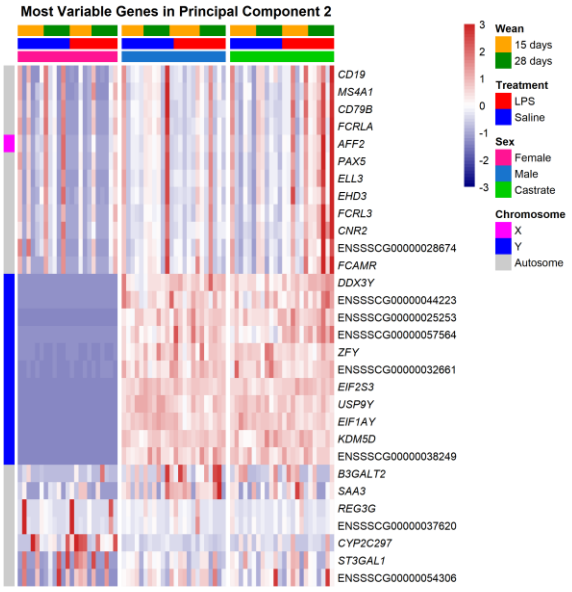

Table 2.1. The number of differentially expressed genes (DEGs) and intersecting genes in female, male, and castrated male group, stratified by wean age.

Group	Late wean	Early wean	Intersection
Female	2,217	2,080	787
Male	3,448	3,631	1,636
Castrate	4,568	2,092	1,220
Intersection	813	343	224

The number of DEGs was calculated considering a p-value ≤ 0.05.

Figure 2. Analysis of the 224 genes that are commonly regulated in response to LPS injection. 2A) Heatmap of 224 intersecting DEGS. Expression values are normalized by row, red indicates higher expression, and blue indicates lower expression. Columns are annotated for wean age, treatment, and sex. 2B) Bar plot displaying the enriched pathways for the 224 intersecting genes, red and blue indicate that the pathway was enriched for upregulated and downregulated genes, respectively. This enrichment analysis was performed in g:Profiler website. 2C) STRING network analysis of protein-protein interactions among genes that contribute to the pathway enrichment analysis. The color scale (blue to red) represents the log2 fold change of the gene, with blue indicating higher abundance in saline-treated samples and red indicating higher abundance in LPS-treated samples.

Figure 2A

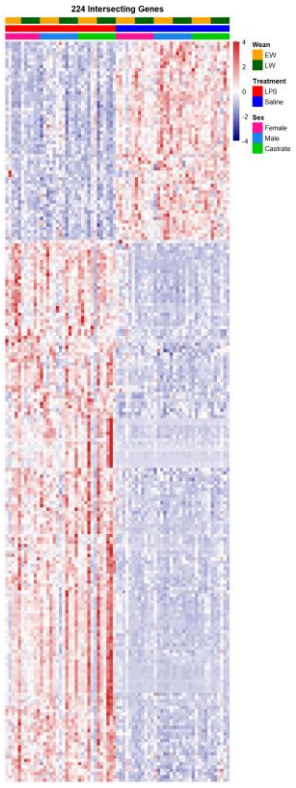

Figure 2 (cont'd)

Figure 2B

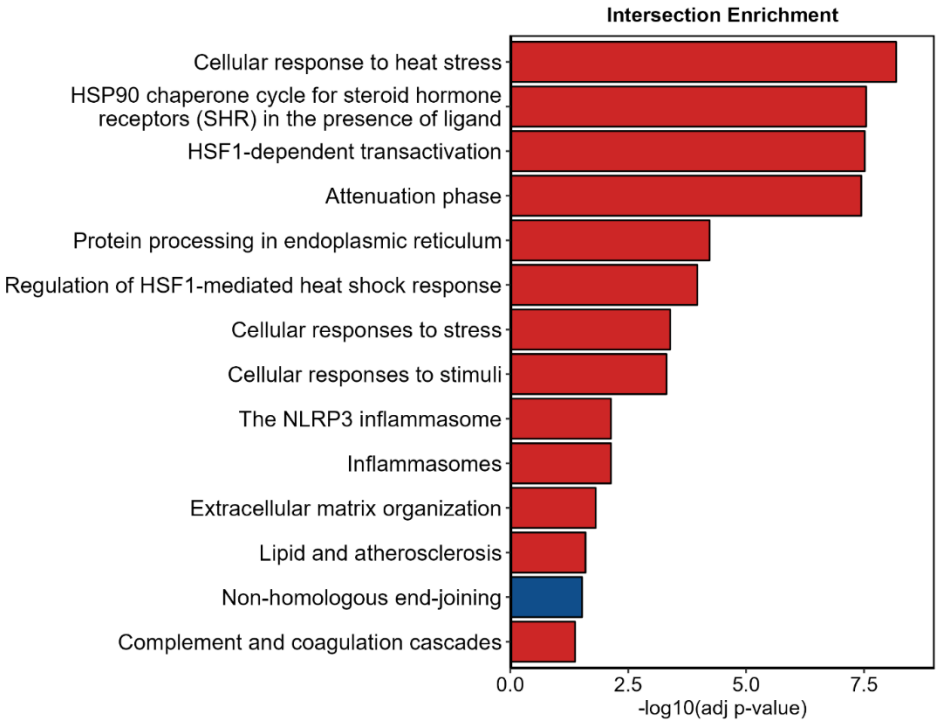

Figure 2C

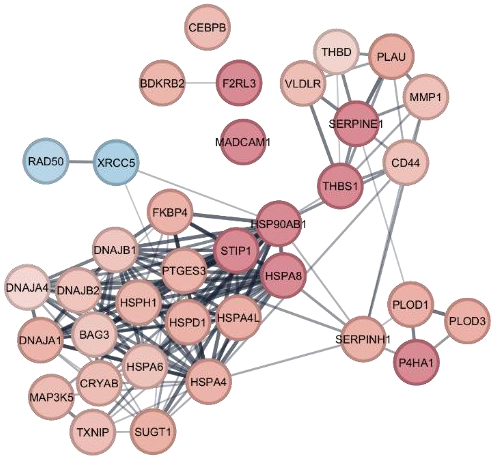

Table 2.2. Differential expression patterns of chaperone genes in female, male, and castrated male pigs in late- and early-wean conditions in response to LPS (log_2foldchange^{Significance}).

	Late Wean			Early Wean		
	Female	**Male**	**Castrate**	**Female**	**Male**	**Castrate**
HSPA8	1.30***	1.92***	1.66***	1.25*	1.95***	1.52***
HSP90AB1	1.02 **	1.25 ***	1.58 ***	0.88*	1.03 ***	1.04 **
FKBP4	1.16*	1.87 ***	1.99 ***	0.80	1.41 ***	1.16*
PTGES3	0.80*	0.90**	1.30 ***	0.50	0.78*	0.75
STIP1	1.41**	2.13 ***	2.27 ***	1.14	1.90 ***	1.55 ***

Adjusted p-value * ≤0.05, **≤0.01, *** ≤0.001

Figure 3. Differential gene expression and gene set enrichment analysis in late-weaned and early-weaned female pigs. A-B) Volcano plots highlighting the top 20 most significantly differentially expressed genes (DEGs) in late-weaned (A) and early-weaned (B) female pigs. Genes with increased expression levels are shown in red, and those with decreased levels are in blue. The dotted line indicates the significance threshold at p-value < 0.05. C-D) Bar plots illustrating the top 5 positively enriched (red) and 5 negatively enriched (blue) gene sets in late-weaned (C) and early-weaned (D) female pigs. Bar height represents the number of genes contributing to each gene set. E-G) Heatmaps showing leading edge genes associated with the top gene sets negatively enriched in late-weaned (E) and early-weaned (F) and positively enriched gene sets in early-weaned (G).

Figure 3A-B

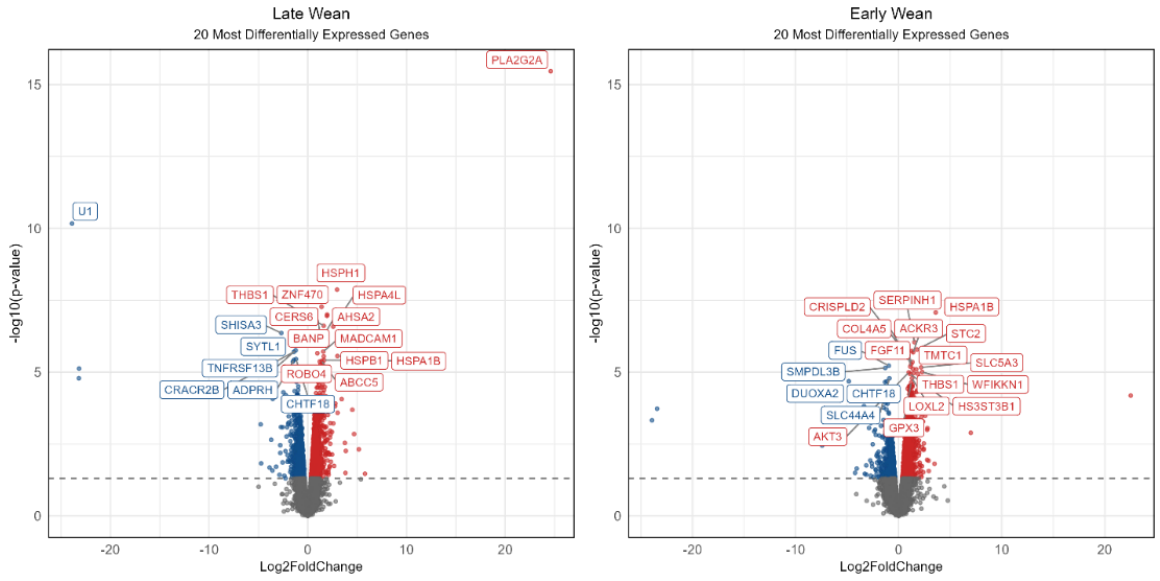

Figure 3 (cont'd)

Figure 3C-D

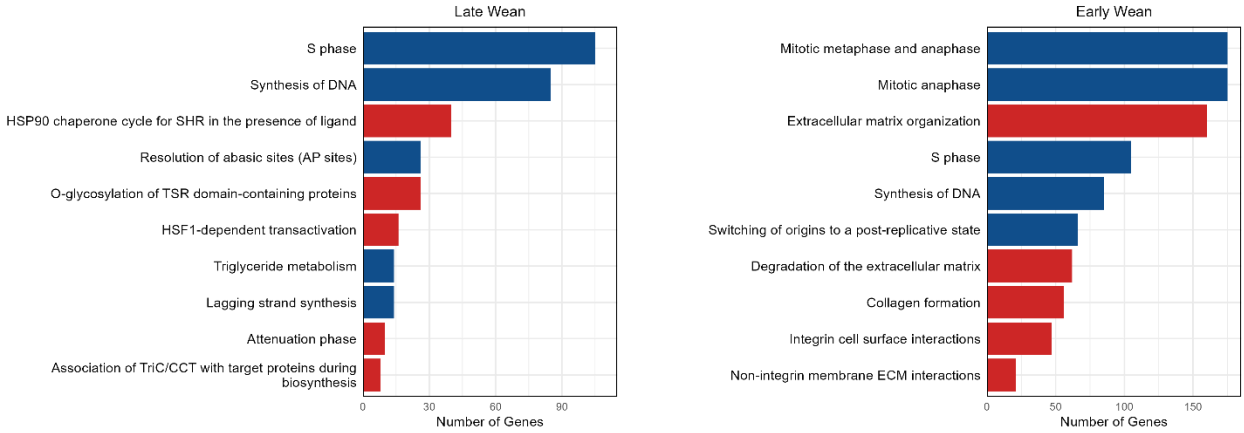

Figure 3E

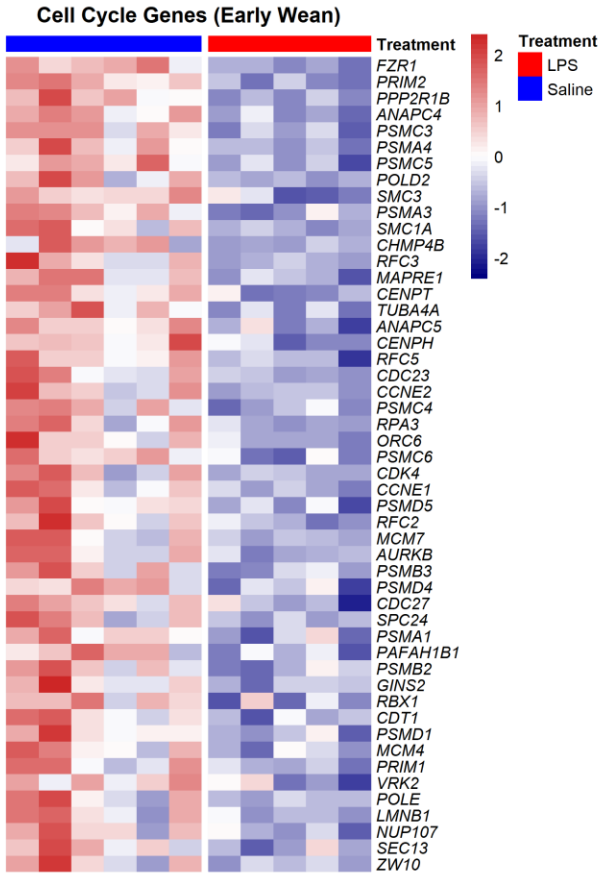

Figure 3 (cont'd)

Figure 3F

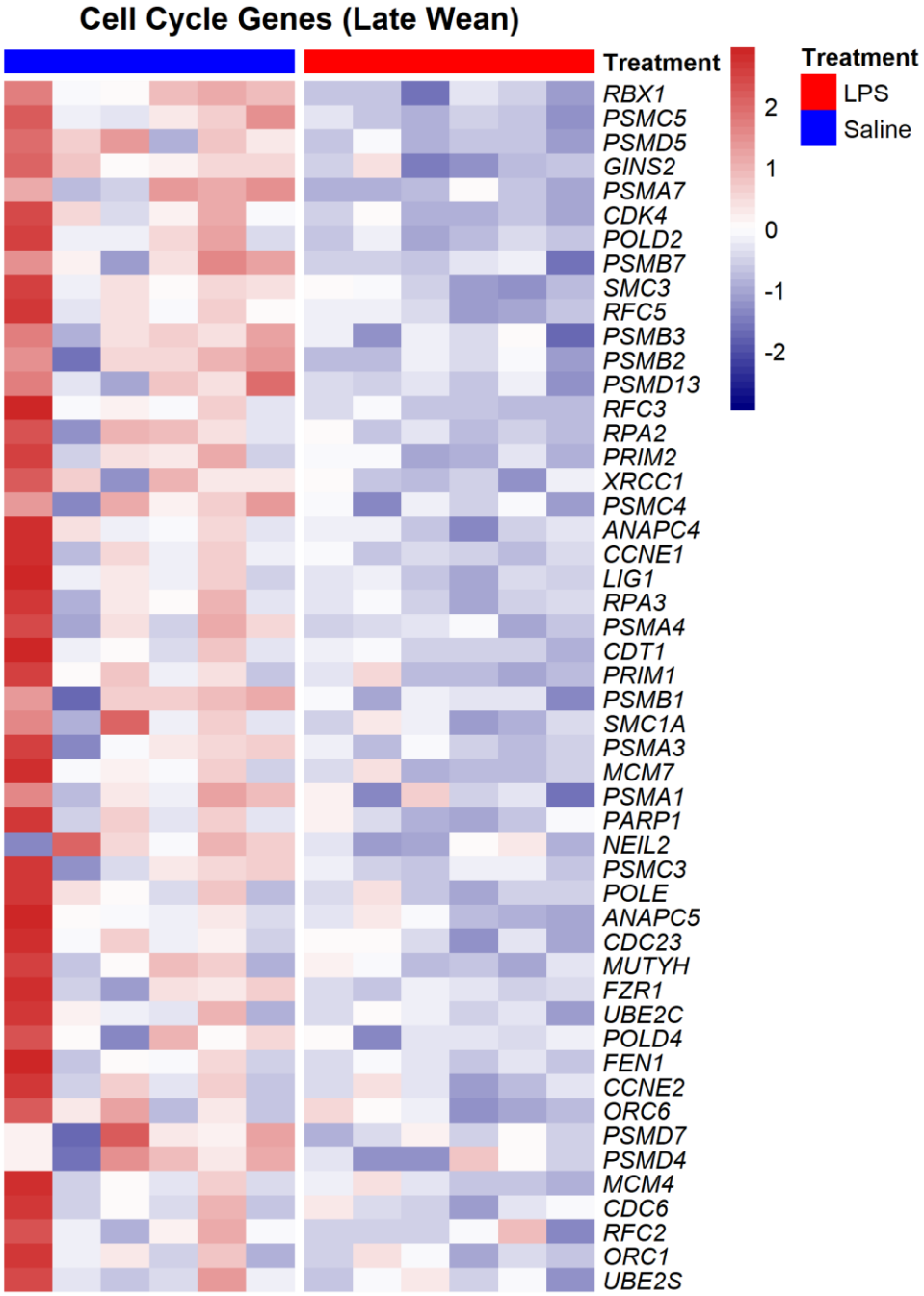

Figure 3(cont'd)

Figure 3G

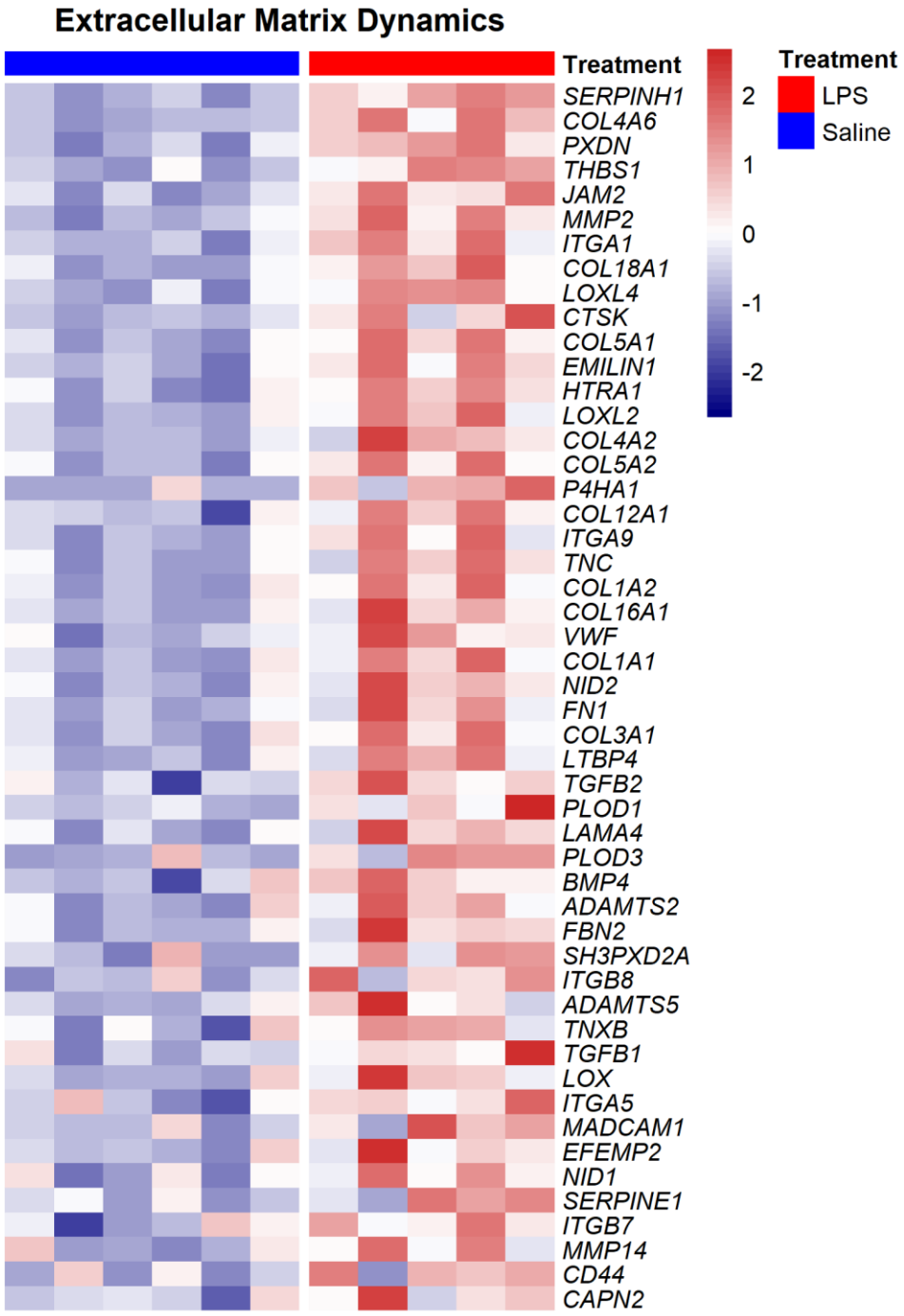

Figure 4. Differential gene expression and gene set enrichment analysis in late-weaned and early-weaned male pigs. A-B) Volcano plots highlighting the top 20 most significantly differentially expressed genes (DEGs) in late- (A) and early-weaned (B) Male pigs. Genes with increased expression levels are shown in red, and those with decreased levels are in blue. The dotted line indicates the significance threshold at $p < 0.05$. C-D) Bar plots illustrating the top 5 positively enriched (red) and 5 negatively enriched (blue) gene sets in late-weaned (C) and early-weaned (D) male pigs. Bar height represents the number of genes contributing to each gene set. E-F) Heatmaps showing leading edge genes associated with the top gene sets positively enriched in late-weaned (E) and early-weaned (F).

***Figure* 4A-B**

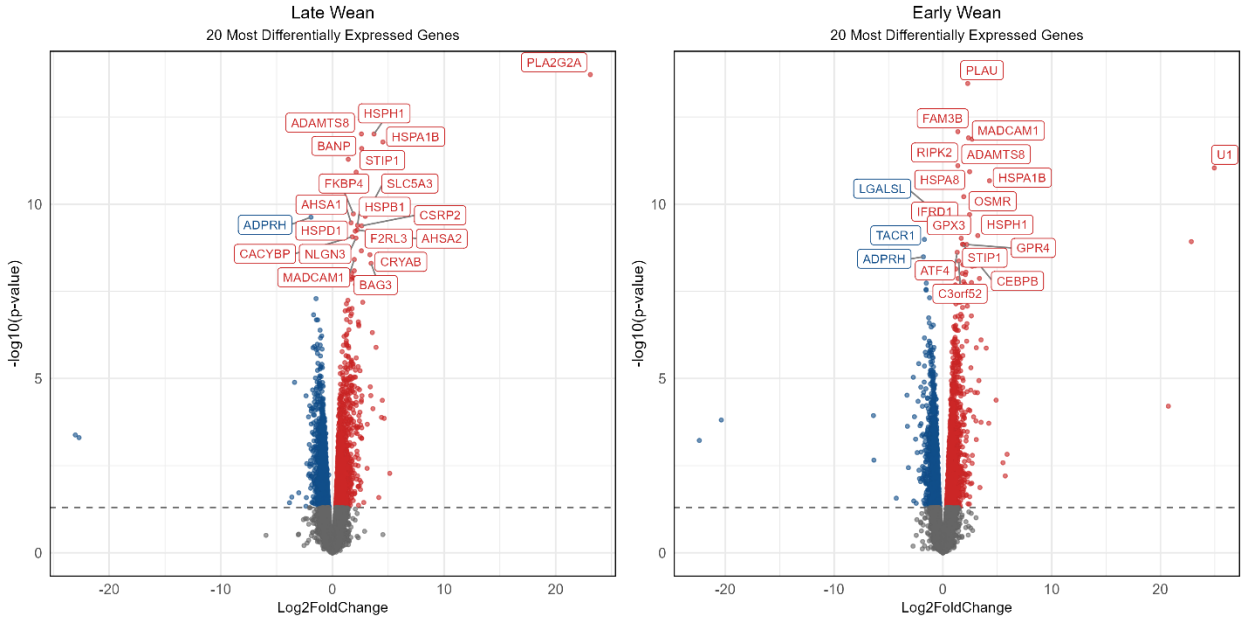

Figure 4 (cont'd)

Figure 4C-D

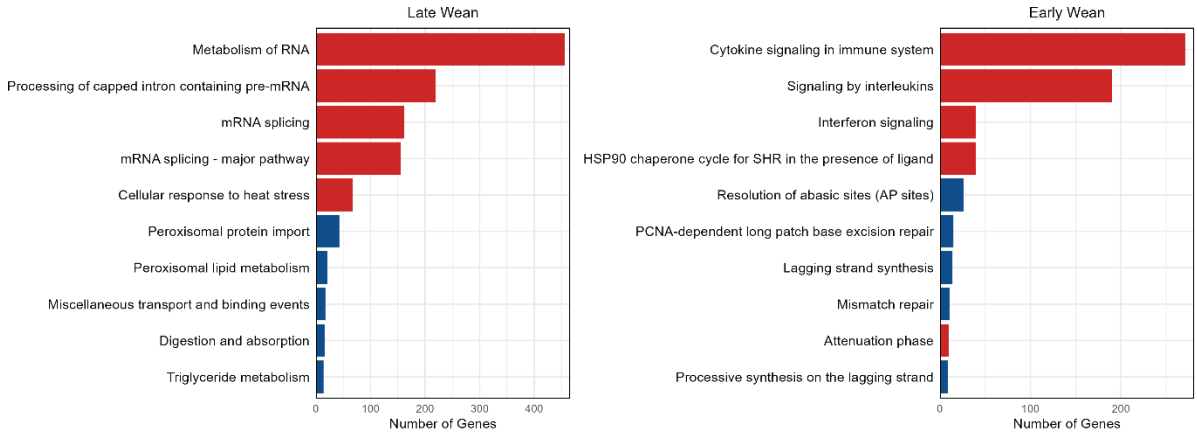

Figure 4 (cont'd)

Figure 4E

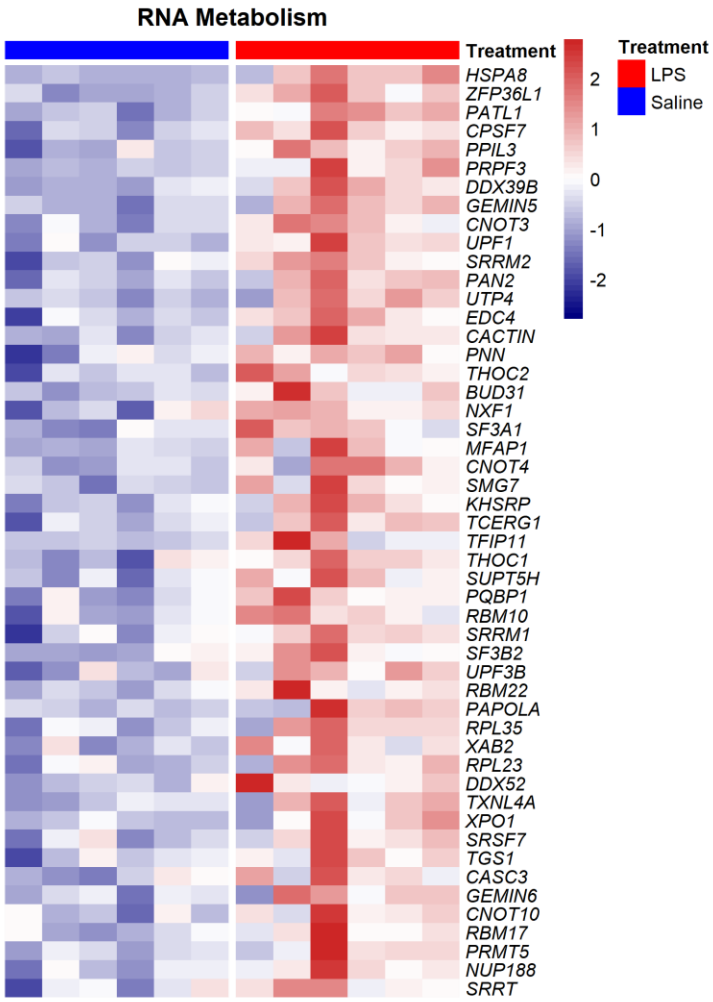

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Figure 4F

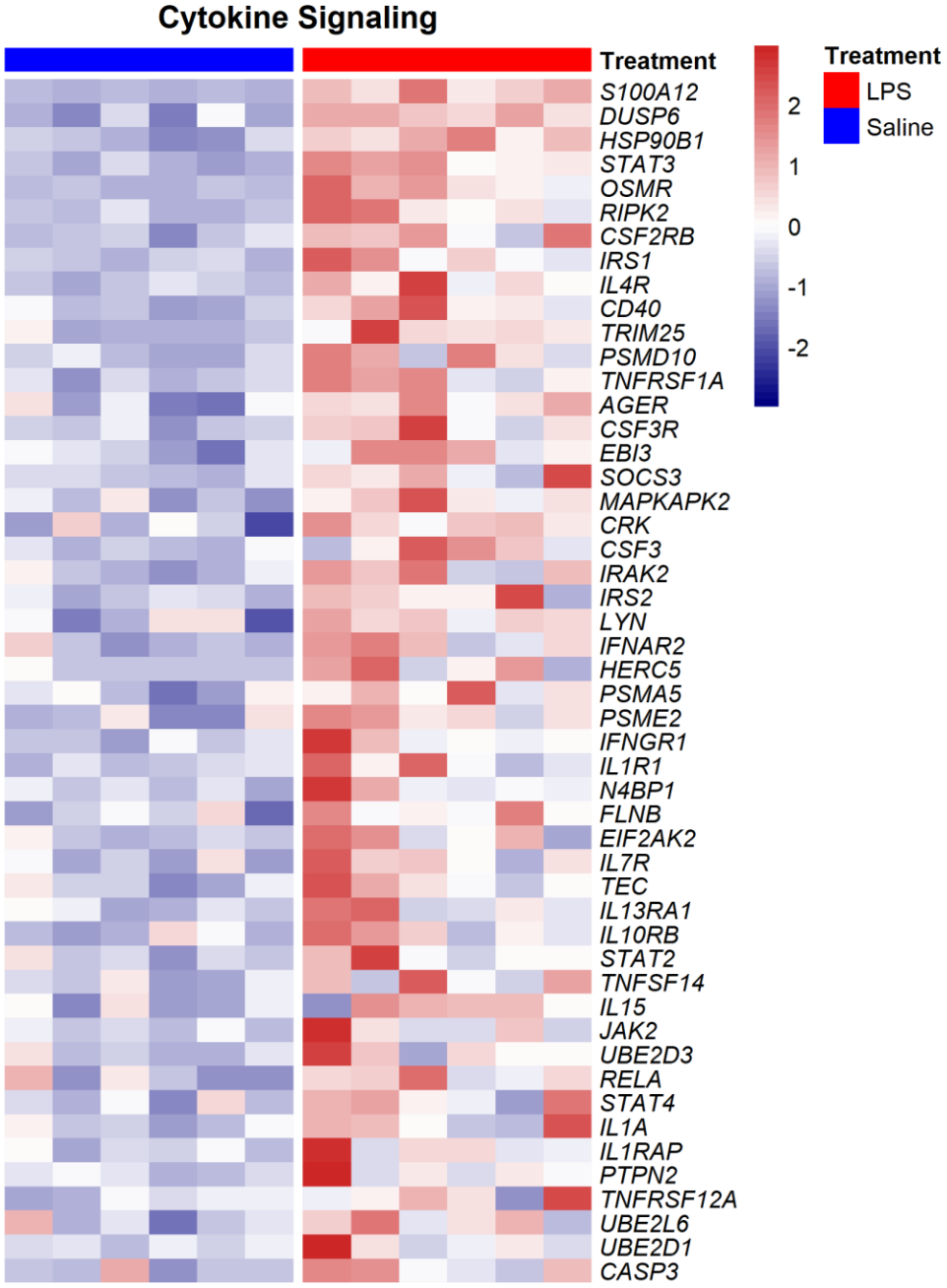


Figure 5. Differential Gene Expression and Gene Set Enrichment Analysis in Late-Weaned and Early-Weaned Castrated Male Pigs. A-B) Volcano plots highlighting the top 20 most significantly differentially expressed genes (DEGs) in late- (A) and early-weaned (B) castrated male pigs. Genes with increased expression levels are shown in red, and those with decreased levels are in blue. The dotted line indicates the significance threshold at $p < 0.05$. C-D) Bar plots illustrating the top 5 positively enriched (red) and 5 negatively enriched (blue) gene sets in late-weaned (C) and early-weaned (D) castrated male pigs. Bar height represents the number of genes contributing to each gene set. E-F) Heatmaps showing leading edge genes associated with the top gene sets positively enriched in late-weaned (E) and early-weaned (F).

Figure 5A-B

Figure 5 (cont'd)

Figure 5C-D

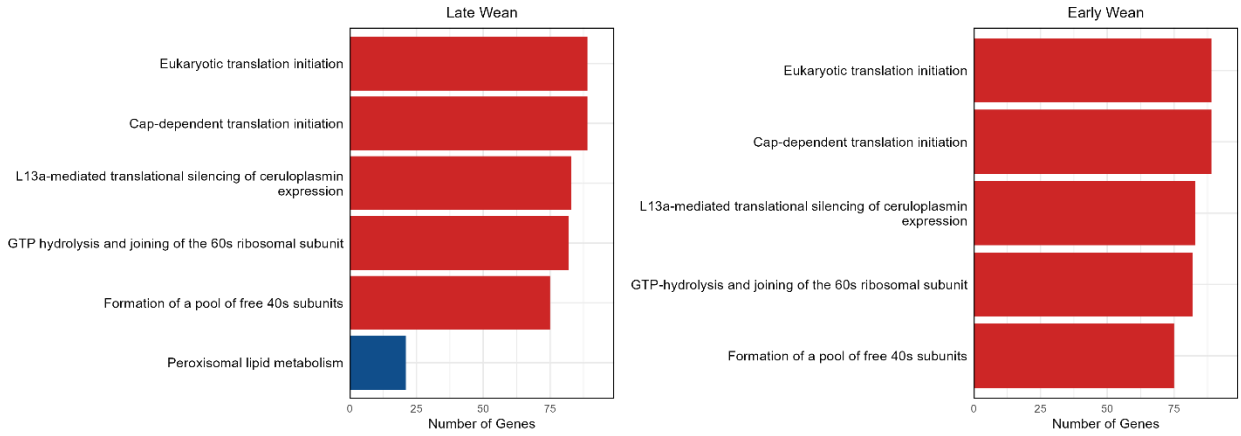


Figure 5E

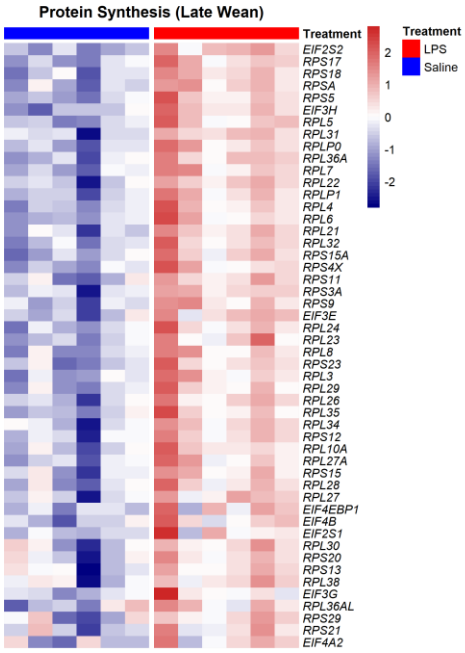


Figure 5 (cont'd)

Figure 5F

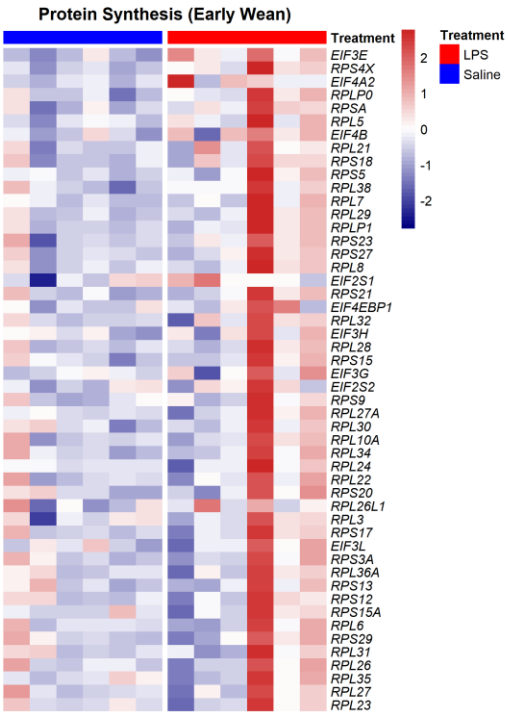


Figure 6. Gene set enrichment analysis (GSEA) of gene sets enriched in late-weaned and early-weaned pigs stratified by sex and castration status. The network analysis was created using EnrichmentMAP, an application in Cytoscape. A-B) Venn diagram of the gene sets in late-weaned group (A) and early-wean group (B). C-D) Network analysis of the GSEA results in late-wean groups (C) and early-wean group (D). Positive enrichment is represented by red squares, negative enrichment is represented by blue squares. Circles represent the enriched gene sets, and the size of the circle indicates the number of genes in the set. The color of the circle corresponds to the group: female pigs (pink), male pigs (blue), and castrated male pigs (green). All gene sets with FDR ≤ 0.05.

Figure 6A

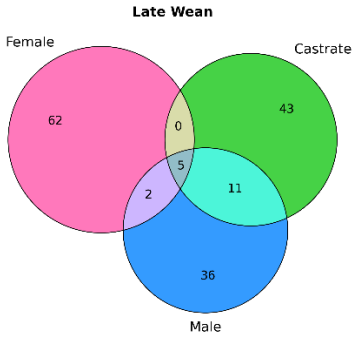


Figure 6B

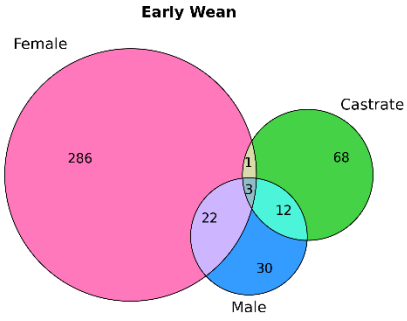


Figure 6 (cont'd)

Figure 6C

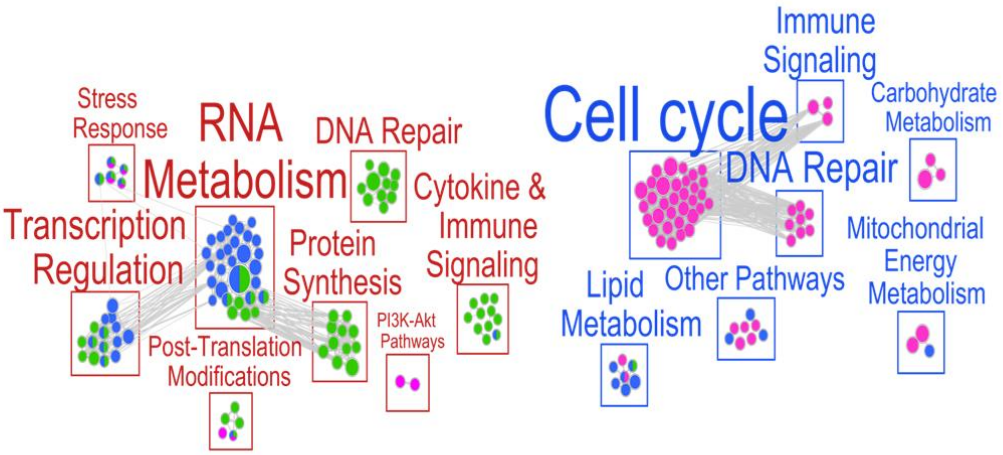


Figure 6D

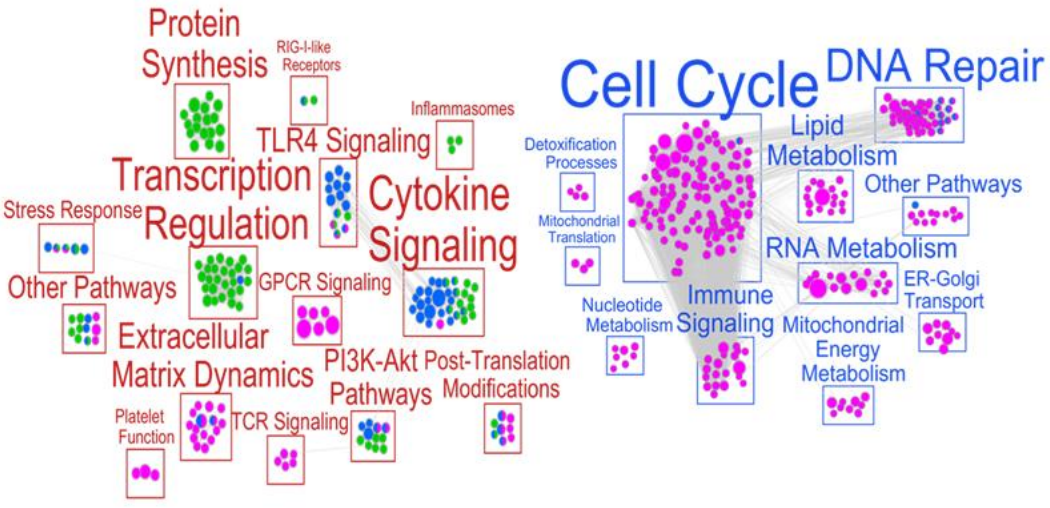
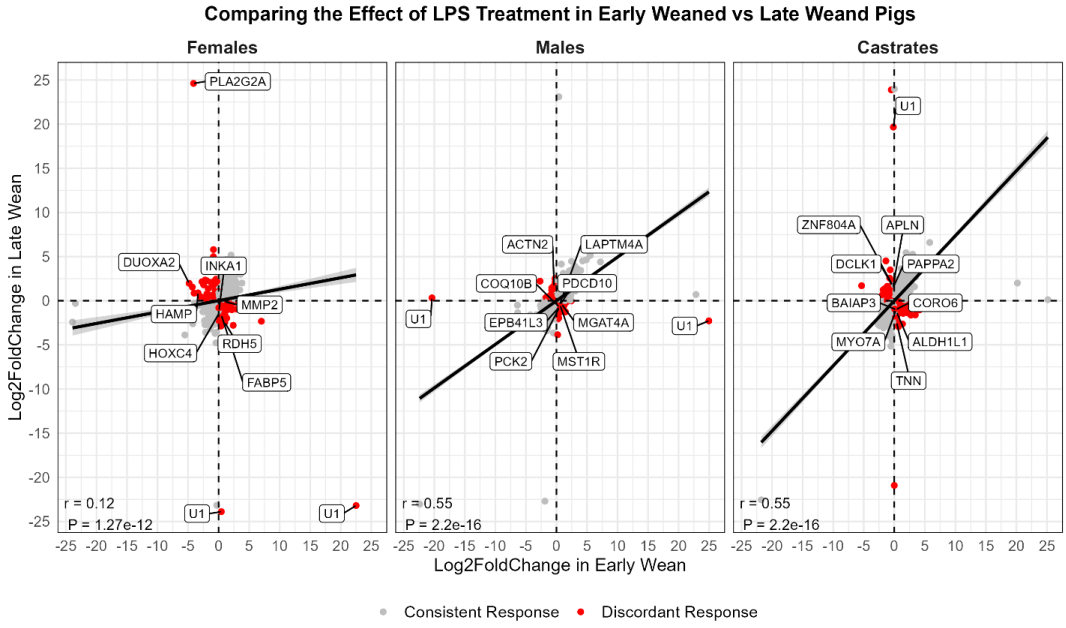


Figure 7. Correlation of differential gene expression in response to LPS treatment between early-weaned and late-weaned female, male, and castrated male groups. Correlations were calculated using the cor.test function in R with Pearson's method to assess the similarity in gene expression changes across weaning ages within each sex. Genes highlighted in red are differently regulated between the two wean groups. Panels show correlations within female, male, and castrated male pigs, with correlation coefficients and p-values on the lower left.



REFERENCES

Agrawal, M., Sabino, J., Frias-Gomes, C., Hillenbrand, C. M., Soudant, C., Axelrad, J. E., Shah, S. C., Ribeiro-Mourão, F., Lambin, T., Peter, I., Colombel, J. F., Narula, N., & Torres, J. (2021). Early life exposures and the risk of inflammatory bowel disease: Systematic review and meta-analyses. *EClinicalMedicine*, *36*. https://doi.org/10.1016/j.eclinm.2021.100884

Alagar Boopathy, L. R., Jacob-Tomas, S., Alecki, C., & Vera, M. (2022). Mechanisms tailoring the expression of heat shock proteins to proteostasis challenges. In *Journal of Biological Chemistry* (Vol. 298, Issue 5). American Society for Biochemistry and Molecular Biology Inc. https://doi.org/10.1016/j.jbc.2022.101796

Ambade, A., Catalano, D., Lim, A., & Mandrekar, P. (2012). Inhibition of heat shock protein (molecular weight 90 kDa) attenuates proinflammatory cytokines and prevents lipopolysaccharide-induced liver injury in mice. *Hepatology*, *55*(5), 1585–1595. https://doi.org/10.1002/hep.24802

Asmal, M., Colgan, J., Naef, F., Yu, B., Lee, Y., Magnasco, M., Luban, J. (2003). Production of Ribosome Components in Effector CD4 T Cells Is Accelerated by TCR Stimulation and Coordinated by ERK-MAPK. *Immunity, 19(4):535-48. 10.1016/s1074-7613(03)00268-1.*

Baker, J. D., Ozsan, I., Ospina, S. R., Gulick, D., & Blair, L. J. (2019). Hsp90 heterocomplexes regulate steroid hormone receptors: From stress response to psychiatric disease. In *International Journal of Molecular Sciences* (Vol. 20, Issue 1). MDPI AG. https://doi.org/10.3390/ijms20010079

Baldwin, J. R., Arseneault, L., Caspi, A., Fisher, H. L., Moffitt, T. E., Odgers, C. L., Pariante, C., Ambler, A., Dove, R., Kepa, A., Matthews, T., Menard, A., Sugden, K., Williams, B., & Danese, A. (2018). Childhood victimization and inflammation in young adulthood: A genetically sensitive cohort study. *Brain, Behavior, and Immunity*, *67*, 211–217. https://doi.org/10.1016/j.bbi.2017.08.025

Baumeister, D., Akhtar, R., Ciufolini, S., Pariante, C. M., & Mondelli, V. (2016). Childhood trauma and adulthood inflammation: A meta-analysis of peripheral C-reactive protein, interleukin-6 and tumour necrosis factor-α. *Molecular Psychiatry*, *21*(5), 642–649. https://doi.org/10.1038/mp.2015.67

Cistrome Data Browser Toolkit. (2025, January 22). Retrieved from: http://dbtoolkit.cistrome.org/?specie=hg38&distance=100k&factor=factor&keyword=chr1%3A19975430%3A19979658%3ANM_001161729%3APLA2G2A#plot_result

Dunn, S. E., Perry, W. A., & Klein, S. L. (2024). Mechanisms and consequences of sex differences in
immune responses. In *Nature Reviews Nephrology* (Vol. 20, Issue 1, pp. 37–55). Nature Research. https://doi.org/10.1038/s41581-023-00787-w

Fardisi, M., Thelen, K., Groenendal, A., Rajput, M., Sebastian, K., Contreras, G. A., & Moeser, A. J. (2023). Early weaning and biological sex shape long-term immune and metabolic responses in pigs. *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-023-42553-9

Forsyth, K. S., Jiwrajka, N., Lovell, C. D., Toothacre, N. E., & Anguera, M. C. (2024). The conneXion between sex and immune responses. In *Nature Reviews Immunology* (Vol. 24, Issue 7, pp. 487–502). Nature Research. https://doi.org/10.1038/s41577-024-00996-9

Gu, Y., Harley, I. T. W., Henderson, L. B., Aronow, B. J., Vietor, I., Huber, L. A., Harley, J. B., Kilpatrick, J. R., Langefeld, C. D., Williams, A. H., Jegga, A. G., Chen, J., Wills-Karp, M., Arshad, S. H., Ewart, S. L., Thio, C. L., Flick, L. M., Filippi, M. D., Grimes, H. L., Karp, C. L. (2009). Identification of IFRD1 as a modifier gene for cystic fibrosis lung disease. *Nature*, *458*(7241), 1039–1042. https://doi.org/10.1038/nature07811

Janssen, W. J., Danhorn, T., Harris, C., Mould, K. J., Lee, F.F., Hedin, B. R., D`Alessandro, A., Leach, S. M., Alper, S. (2020). Inflammation-Induced Alternative Pre-mRNA Splicing in Mouse Alveolar Macrophages. *G3 Genes|Genomes|Genetics*, 10(2), 555-567. https://doi.org/10.1534/g3.119.400935

Kuhlman, K. R., Horn, S. R., Chiang, J. J., & Bower, J. E. (2020). Early life adversity exposure and circulating markers of inflammation in children and adolescents: A systematic review and meta-analysis. *Brain, Behavior, and Immunity*, *86*, 30–42. https://doi.org/10.1016/j.bbi.2019.04.028

Laffont, S., Blanquart, E., Savignac, M., Cénac, C., Laverny, G., Metzger, D., Girard, J. P., Belz, G. T., Pelletier, L., Seillet, C., & Guéry, J. C. (2017). Androgen signaling negatively controls group 2 innate lymphoid cells. *Journal of Experimental Medicine*, *214*(6), 1581–1592. https://doi.org/10.1084/jem.20161807

Lai, H. C., Ho, U. Y., James, A., Souza, P. D., Robersts, T. L. (2021). RNA Metabolism and Links to Inflammatory Regulation and Disease. Cellular and Molecular Like Sciences, 79(21). https://doi.org/10.1007/s00018-021-04073-5.

Lelouard, H., Schmidt, E. K., Camosseto, V., Clavarino, G., Ceppi, M., Hsu, H. T., & Pierre, P. (2007). Regulation of translation is required for dendritic cell function and survival during activation. *Journal of Cell Biology*, *179*(7), 1427–1439. https://doi.org/10.1083/jcb.200707166

Mackey, E., Thelen, K. M., Bali, V., Fardisi, M., Trowbridge, M., Jordan, C. L., & Moeser, A. J. (2020). *Perinatal androgens organize sex differences in mast cells and attenuate anaphylaxis severity into adulthood*. https://doi.org/10.1073/pnas.1915075117/-/DCSupplemental

Medland, J. E., Pohl, C. S., Edwards, L. L., Frandsen, S., Bagley, K., Li, Y., & Moeser, A. J. (2016). Early life adversity in piglets induces long-term upregulation of the enteric cholinergic nervous system and heightened, sex-specific secretomotor neuron responses. *Neurogastroenterology and Motility*, *28*(9), 1317–1329. https://doi.org/10.1111/nmo.12828

Merrick, M. T., Ford, D. C., Ports, K. A., Guinn, A. S., Chen, ; Jieru, Klevens, J., Metzler, M., Jones, C. M., Simon, T. R., Daniel, V. M., Ottley, P., & Mercy, J. A. (n.d.). *Vital Signs: Estimated Proportion of Adult Health Problems Attributable to Adverse Childhood Experiences and Implications for Prevention — 25 States, 2015–2017*. https://www.cdc.gov/brfss.

Meurens, F., Summerfield, A., Nauwynck, H., Saif, L., & Gerdts, V. (2012). The pig: A model for human infectious diseases. In *Trends in Microbiology* (Vol. 20, Issue 1, pp. 50–57). https://doi.org/10.1016/j.tim.2011.11.002

Moeser, A. J., Pohl, C. S., & Rajput, M. (2017). Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. In *Animal Nutrition* (Vol. 3, Issue 4, pp. 313–321). KeAi Communications Co. https://doi.org/10.1016/j.aninu.2017.06.003

Monestier, O., & Blanquet, V. (2016). WFIKKN1 and WFIKKN2: "Companion" proteins regulating TGFB activity. In *Cytokine and Growth Factor Reviews* (Vol. 32, pp. 75–84). Elsevier Ltd. https://doi.org/10.1016/j.cytogfr.2016.06.003

Morales, J., Dereu, A., Manso, A., de Frutos, L., Piñeiro, C., Manzanilla, E. G., & Wuyts, N. (2017). Surgical castration with pain relief affects the health and productive performance of pigs in the suckling period. *Porcine Health Management*, *3*. https://doi.org/10.1186/s40813-017-0066-1

Pohl, C. S., Medland, J. E., Mackey, E., Edwards, L. L., Bagley, K. D., DeWilde, M. P., Williams, K. J., & Moeser, A. J. (2017). Early weaning stress induces chronic functional diarrhea, intestinal barrier defects, and increased mast cell activity in a porcine model of early life adversity. *Neurogastroenterology and Motility*, *29*(11). https://doi.org/10.1111/nmo.13118

Ramos-Casals, M., Brito-Zerón, P., Kostov, B., Sisó-Almirall, A., Bosch, X., Buss, D., Trilla, A., Stone, J. H., Khamashta, M. A., & Shoenfeld, Y. (2015). Google-driven search for big data in autoimmune geoepidemiology: Analysis of 394,827 patients with systemic autoimmune diseases. In *Autoimmunity Reviews* (Vol. 14, Issue 8, pp. 670–679). Elsevier B.V. https://doi.org/10.1016/j.autrev.2015.03.008

Ren, Q., Liu, Z., Wu, L., Yin, G., Xie, X., Kong, W., Zhou, J., & Liu, S. (2023). C/EBPβ: The structure, regulation, and its roles in inflammation-related diseases. In *Biomedicine and Pharmacotherapy* (Vol. 169). Elsevier Masson s.r.l. https://doi.org/10.1016/j.biopha.2023.115938

Rettew, J. A., Huet, Y. M., & Marriott, I. (2009). Estrogens augment cell surface TLR4 expression on murine macrophages and regulate sepsis susceptibility in vivo. *Endocrinology*, *150*(8), 3877–3884. https://doi.org/10.1210/en.2009-0098

Rettew, J. A., Huet-Hudson, Y. M., & Marriott, I. (2008). Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. *Biology of Reproduction*, *78*(3), 432–437. https://doi.org/10.1095/biolreprod.107.063545

Rosini, S., Pugh, N., Bonna, A. M., Hulmes, D. J. S., Farndale, R. W., & Adams, J. C. (2018).

Thrombospondin-1 promotes matrix homeostasis by interacting with collagen and lysyl oxidase precursors and collagen cross-linking sites. In *Sci. Signal* (Vol. 11). http://stke.sciencemag.org/

Schaaf, C. R., & Gonzalez, L. M. (2022). Use of Translational, Genetically Modified Porcine Models to Ultimately Improve Intestinal Disease Treatment. In *Frontiers in Veterinary Science* (Vol. 9). Frontiers Media S.A. https://doi.org/10.3389/fvets.2022.878952

Schewe, M., Franken, P. F., Sacchetti, A., Schmitt, M., Joosten, R., Böttcher, R., van Royen, M. E., Jeammet, L., Payré, C., Scott, P. M., Webb, N. R., Gelb, M., Cormier, R. T., Lambeau, G., & Fodde, R. (2016). Secreted Phospholipases A2 Are Intestinal Stem Cell Niche Factors with Distinct Roles in Homeostasis, Inflammation, and Cancer. *Cell Stem Cell*, *19*(1), 38–51. https://doi.org/10.1016/j.stem.2016.05.023

Shi, J., Sun, S., Liao, Y., Tang, J., Xu, X., Qin, B., Qin, C., Peng, L., Luo, M., Bai, L., & Xie, F. (2019). Advanced oxidation protein products induce G1 phase arrest in intestinal epithelial cells via a RAGE/CD36-JNK-p27kip1 mediated pathway. *Redox Biology*, *25*. https://doi.org/10.1016/j.redox.2019.101196

Shin, D., Chang, S. Y., Bogere, P., Won, K. H., Choi, J. Y., Choi, Y. J., Lee, H. K., Hur, J., Park, B. Y., Kim, Y., & Heo, J. (2019). Beneficial roles of probiotics on the modulation of gut microbiota and immune response in pigs. *PLoS ONE*, *14*(8). https://doi.org/10.1371/journal.pone.0220843

Shinton, S. A., Brill-Dashoff, J., & Hayakawa, K. (2022). Pla2g2a promotes innate Th2-type immunity lymphocytes to increase B1a cells. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-18876-4

Stewart, D. C., Berrie, D., Li, J., Liu, X., Rickerson, C., Mkoji, D., Iqbal, A., Tan, S., Doty, A. L., Glover, S. C., & Simmons, C. S. (2018). Quantitative assessment of intestinal stiffness and associations with fibrosis in human inflammatory bowel disease. *PLoS ONE*, *13*(7). https://doi.org/10.1371/journal.pone.0200377

Taslem Mourosi, J., Anwar, S., & Hosen, M. J. (2022). The sex and gender dimensions of COVID-19: A narrative review of the potential underlying factors. In *Infection, Genetics and Evolution* (Vol. 103). Elsevier B.V. https://doi.org/10.1016/j.meegid.2022.105338

Weiner, J., Obermayer, B., & Beule, D. (2022). Venn Diagrams May Indicate Erroneous Statistical Reasoning in Transcriptomics. *Frontiers in Genetics*, *13*. https://doi.org/10.3389/fgene.2022.818683

Yu, X., Li, S., Xu, Y., Zhang, Y., Ma, W., Liang, C., Lu, H., Ji, Y., Liu, C., Chen, D., & Li, J. (2020). Androgen Maintains Intestinal Homeostasis by Inhibiting BMP Signaling via Intestinal Stromal Cells. *Stem Cell Reports*, *15*(4), 912–925. https://doi.org/10.1016/j.stemcr.2020.08.001

Zhou, W., Davis, E. A., Li, K., Nowak, R. A., & Dailey, M. J. (2018). Sex differences influence intestinal epithelial stem cell proliferation independent of obesity. *Physiological Reports*, *6*(13).

https://doi.org/10.14814/phy2.13746

APPENDIX

Figure S1. Sequencing coverage per sample.

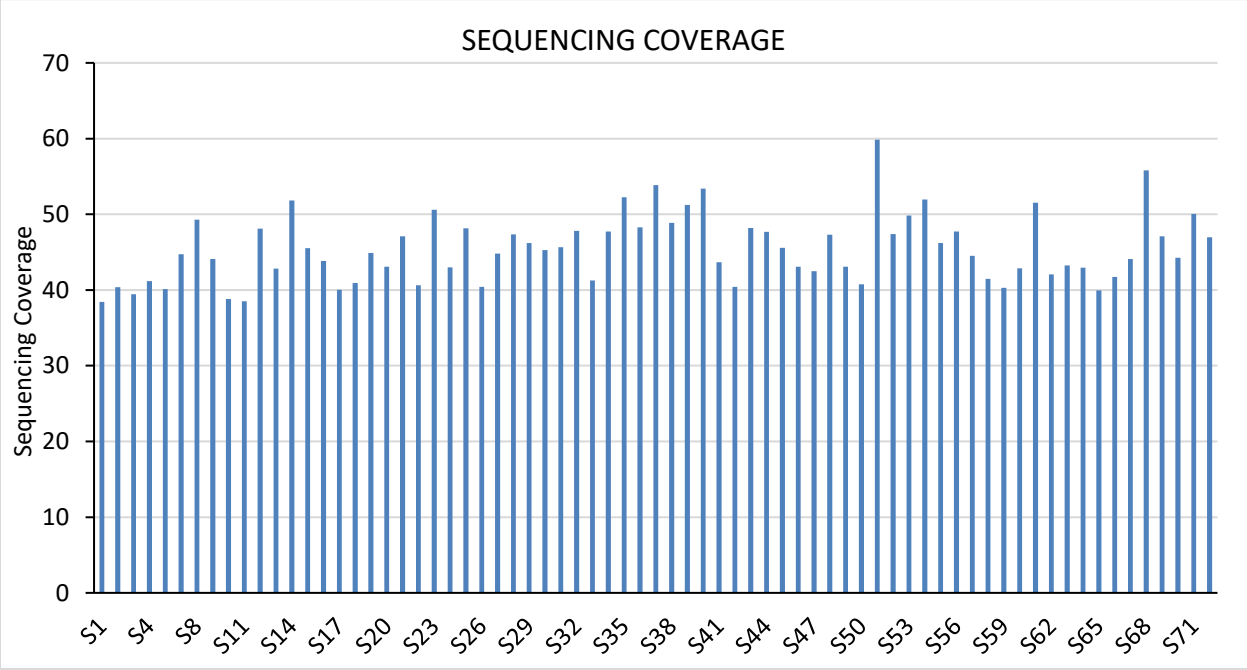


Figure S2. Enrichment analysis of the 3,348 genes exclusively differentially expressed in the late-wean castrated male group.

Figure S2A

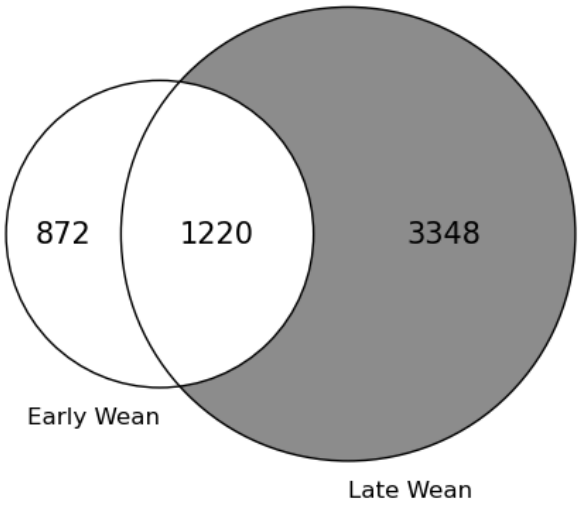


Figure S2 (cont'd)

Figure S2B