



MULTIVARIATE DEPENDENCE IN HORMONAL AND IMMUNE RESPONSES: EFFECT ESTIMATION AND PHYSIOLOGICAL VARIATION

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Abstract

This study examined multivariate dependence between hormonal biomarkers and immune responses, emphasizing effect estimation and physiological variation. Participant-level data from healthy adults were analyzed using progesterone, testosterone, 25-hydroxy vitamin D3, and lipopolysaccharide-stimulated IFN-gamma, IL-1beta, IL-6, and TNF-alpha responses. Descriptive statistics, Spearman correlation, false-discovery-rate adjustment, Mann-Whitney U tests, and multivariable linear regression with heteroskedasticity-robust standard errors were applied. Regression models adjusted for age, sex, body mass index, smoking status, and the remaining hormonal variables. Testosterone showed significant positive unadjusted correlations with all four cytokines, whereas progesterone showed minimal associations and vitamin D3 demonstrated a modest inverse relationship with IL-6. However, none of the hormonal coefficients remained statistically significant after multivariable adjustment and correction for multiple testing. Pronounced sex-related physiological variation was observed, with males exhibiting markedly higher testosterone concentrations and stronger IFN-gamma, IL-1beta, IL-6, and TNF-alpha responses, while females showed higher vitamin D3 concentrations. These findings indicate that apparent hormone-immune relationships are strongly influenced by underlying physiological heterogeneity and may attenuate substantially after adjustment for major covariates. Overall, multivariate analytical approaches provide a more comprehensive framework for evaluating endocrine-immune interactions and distinguishing direct biomarker relationships from broader physiological variation.

Keywords: Hormonal biomarkers; Immune response; Cytokines; Physiological variation; Multivariate analysis

1. Introduction

Hormonal and immune system processes are not independent physiological subsystems. Endocrine activity may modulate inflammatory activity, cytokine production, metabolism, and the function of immune cells, and immune activity may modulate endocrine activity. Therefore, analyzing separate biomarkers can miss common biological variation and inter-individual variations. Multivariate approaches are especially useful since complex physiological states may arise from coordinated variations of multiple biological pathways, rather than from a single marker. The importance of considering physiological responses as multivariate phenotypes with interacting endocrine, immune, and behavioural components has also been stressed in the field of stress research (Telemeco & Gangloff, 2020).

Endocrine-immune communication is part of the process of the sex hormones. Experimental and clinical studies show that female sex hormones have the ability to influence behavioural and physiological immune parameters, which indicates that hormones play a role in variation in immune responsiveness (Keller & Diekhof, 2024). Sex is thus an important analytical variable in immunological research as sex-specific differences in gonadal hormones, chromosomes, metabolism, and immune-cell regulation may result in sex-specific inflammatory profiles (Dakup et al., 2022). Sex-dependent immune responses were also underscored in the COVID-19 pandemic, as both antiviral immunity and inflammatory regulation were found to be different between males and females (Gadi et al., 2020). The mechanisms proposed for how sex plays a role in infective disease outcomes also emphasize the need to take both endocrine and immunological factors into account (Wray & Arrowsmith, 2021).

Other physiological and behavioural factors also influence endocrine-immune relationships. Habitual physical activity has been associated with changes in thyroid hormones, inflammatory markers and immune system markers, highlighting the interrelatedness of endocrine, metabolic and immune regulation (Klasson et al., 2022). Another factor related to the endocrine system that has known immunomodulatory properties is vitamin D, which has been shown to differ in circulating levels and was linked to differences in innate and adaptive immunity (Wimalawansa, 2022). These types of data can be used to explore vitamin D in addition to steroid hormones in the characterization of cytokine response variability.

Immune heterogeneity is another significant factor that is linked to age. Peripheral blood immune cell composition and functional states have been extensively altered in humans as a result of aging, as seen by high dimensional analyses of peripheral blood cells, especially in the context of aging and frailty (Luo et al., 2022). Other developmental transitions, such as adolescence, may also bring about endocrine and immune changes. Previous studies have shown that during puberty, transcriptional changes in immune pathways are associated with developmental maturation in a longitudinal analysis (Resztak et al., 2023). Such results show the need to consider the physiological factors, including age, sex, and body composition, when considering endocrine-immune dependence.

Stress-related regulatory pathways are also found in the endocrine and immune systems. Long-term effects of prenatal exposure to cytokines on neural circuits underlying stress regulation have been observed, and there is evidence that sex moderates the influence of inflammatory exposure on neuroendocrine function (Goldstein et al., 2021). Bidirectional communication between central regulatory systems and immune responses is further demonstrated by the fact that behaviourally mediated neural mechanisms can influence humoral immunity (Zhang et al., 2020). Further, naturalistic multivariate studies have also demonstrated that emotional states can account for changes in cellular immune activity across time (Seizer et al., 2023). Another example of physiological complexity with individual variability is age-dependent interactions between exercise, endocrine regulation, inflammation, and psychological processes (Xue et al., 2025).

Although these interrelated mechanisms have become recognised, there have been fewer studies that quantify the dependence among the circulating hormonal biomarkers and the experimental induced cytokine response, whilst simultaneously controlling for important physiological covariates. Thus, the aim of the present study was to identify multivariate dependence between the response of progesterone, testosterone, 25-hydroxy vitamin D3, and LPS-induced cytokines, including IFN-gamma, IL-1beta, IL-6, and TNF-alpha. It comprised descriptive characterization, hormone-immune correlation analysis, adjusted effect estimation and assessment of physiological variation by sex. The aims were to measure hormonal and immune variability, to define important endocrine-immune relationships, to see if these remained significant after controlling for age, sex, BMI and smoking, and to assess the differences in hormonal and cytokine responses between sexes.

2. Methodology

2.1 Study Design and Analytical Sample

A secondary quantitative analysis was performed on measurements that were collected from healthy adult volunteers at the participant level to assess their physiological, hormonal and immune response. Records were matched on the common participant identifier, resulting in a basic analytical sample of 489 participants (278 females and 211 males). The sample size used for each analysis was variable since some biomarkers had missing values. No imputation of observations was performed. Rather, all subjects with complete data for the variables for each descriptive, correlation, group-comparison, and regression analysis were used. For the principal descriptive variables, denominators varied from 473 to 489 in the fully adjusted regression models, while for the latter it ranged from 462 to 468 (ter Horst, 2021).

2.2 Variables and Outcome Definition

The hormones that were measured were mainly progesterone, 25-hydroxy vitamin D3, and testosterone. Main immune response variables were interferon gamma (IFN gamma), interleukin 1 beta (IL-1beta), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF alpha). Cytokine analyses were limited to the levels measured in the 48-hour stimulated whole

blood, as conditions for stimulation were kept comparable. Cytokines levels were measured in pg/mL. Physiological covariates for adjusted analyses included: age, sex, body mass index (BMI), and current smoking status. Additionally, sex was explored as an indicator of physiological differences in hormonal and cytokine responses.

2.3 Descriptive and Hormone-Immune Dependence Analysis

Several variables were found to have skewed distributions and were summarized using the mean, standard deviation, median, first quartile and third quartile for these continuous variables. Denominators were not included for unavailable cases in the analysis of each of the variables. Hormone-immune dependence was assessed in two ways: first, using Spearman rank correlation coefficients (ρ) between each of the three hormonal markers and each of the four LPS-stimulated cytokine responses (12 pairwise associations); and second, by calculating a two-way correlation matrix for the 12 associations listed above. Hormone-immune dependence was assessed in two ways: first, using Spearman rank correlation coefficients (ρ) between each of the three hormonal markers and each of the four LPS-stimulated cytokine responses (12 pairwise associations); and second, by calculating a two-way correlation matrix for the 12 associations listed above. Observations reported for individual correlations were dependent on the availability of the biomarker. For a number of comparisons involving these hormones and cytokines, the raw p-values were adjusted for multiple testing using a false-discovery-rate procedure and the adjusted probabilities reported as FDR q-values. The significance of a value for 'q' was determined at the level of $< .05$.

2.4 Multivariable Effect Estimation

Multivariable linear regression models were fitted separately for IFN-gamma, IL-1beta, IL-6 and TNF-alpha. All cytokine concentrations were transformed with the $\log(1 + x)$ function to decrease the positive skewness of the data and then standardized prior to modelling. The main explanatory variables (progesterone, testosterone, and 25-hydroxy vitamin D3) were entered concurrently and standardized prior to estimation. The two models were additionally adjusted for standardized age, standardized BMI, sex and current smoking status. The regression coefficients were thus reported as standardized beta estimates with 95% confidence intervals. In order to conduct statistical inference heteroskedasticity-robust standard errors were used. A total of 468, 465, 462, and 463 complete case samples were used for IFN-gamma, IL-1beta, IL-6, and TNF-alpha, respectively. The four adjusted models produced 12 hormone coefficients, which were adjusted for FDR and $q < .05$ was considered statistically significant. These estimates were considered as adjusted associations instead of real cause-effect association.

2.5 Assessment of Physiological Variation

Physiological variation according to sex was evaluated for the three hormonal biomarkers and four LPS-stimulated cytokine outcomes. The distributions of these biomarkers were not normal and were highly skewed in several cases, and so Mann-Whitney U tests were performed to compare females and males. The sex-specific medians and interquartile ranges were used for the summary of the results with the rank-biserial correlation coefficient as the effect-size measure. Sample sizes were missing for some of the biomarkers and are therefore presented separately for females and males. False-discovery-rate adjustment was applied to multiple sex-based comparison and FDR q-values $< .05$ were considered statistically significant. To facilitate graphical comparison of the variation of cytokines by sex, cytokine concentration for each outcome was transformed to a log scale and standardized to a common scale.

2.6 Ethical Considerations

This analysis was based on anonymised secondary data from an existing research resource and there was no direct recruitment, intervention, or collection of new biological specimens. Participant identifiers were used only for dataset linkage purposes and analyses were conducted at an aggregate level (not an individual level) and without attempts to identify participants. The study therefore followed the principles of confidentiality, responsible use of secondary data and transparency of reporting.

3. Results

The five source files were joined together by the common identifier of healthy volunteers. The core merged dataset consisted of 489 participants (278 females; 56.9% and 211 males; 43.1%). Cytokine measurements were highly right skewed and medians and interquartile ranges were emphasised in descriptive analyses. To facilitate comparison across immune outcomes, the primary immune-response analysis focused on cytokine production in LPS-stimulated whole blood at 48 h (IFN-gamma, IL-1beta, IL-6, and TNF-alpha).

3.1 Hormonal and Immune-Response Characteristics

There was wide inter-individual variation in both hormonal measures and LPS-induced cytokine responses. The median levels of progesterone, testosterone, and 25-hydroxy vitamin D3 were 0.24, 1.46, and 70.0, respectively (Table 1). The immune measurements with the greatest absolute response were IL-6 (median 9,017 pg/mL), and IL-1beta (median 1,902.5 pg/mL). The median levels of IFN-gamma and TNF-alpha were 338 pg/mL and 903.5 pg/mL, respectively.

Table 1. Descriptive characteristics of physiological, hormonal, and immune-response variables

Variable	N	Median	Q1	Q3	Mean	SD
Age (years)	482	23.00	21.00	26.75	27.47	12.50
BMI (kg/m ²)	473	22.22	20.72	24.28	22.67	2.72
Progesterone	484	0.24	0.17	0.34	1.11	5.02
Testosterone	484	1.46	0.79	16.90	8.86	10.04
25-hydroxy vitamin D3	486	70.00	53.00	87.00	71.44	26.34
LPS IFN-gamma (pg/mL)	489	338.00	152.00	706.00	546.91	631.44
LPS IL-1beta (pg/mL)	486	1,902.50	1,472.75	2,510.00	2,038.50	841.15
LPS IL-6 (pg/mL)	483	9,017.00	7,361.50	11,218.50	9,576.05	3,331.21
LPS TNF-alpha (pg/mL)	484	903.50	596.50	1,271.50	1,028.71	642.76

Note: Q1 and Q3 represent the first and third quartiles, respectively. Sample size varies because of missing biomarker observations.

The distributions of the three hormonal variables are presented in Figure 1. Standardization was used only for graphical comparison because the biomarkers were recorded on different numerical scales. Testosterone displayed substantially greater between-participant dispersion than progesterone, while vitamin D3 exhibited relatively broad physiological variability.

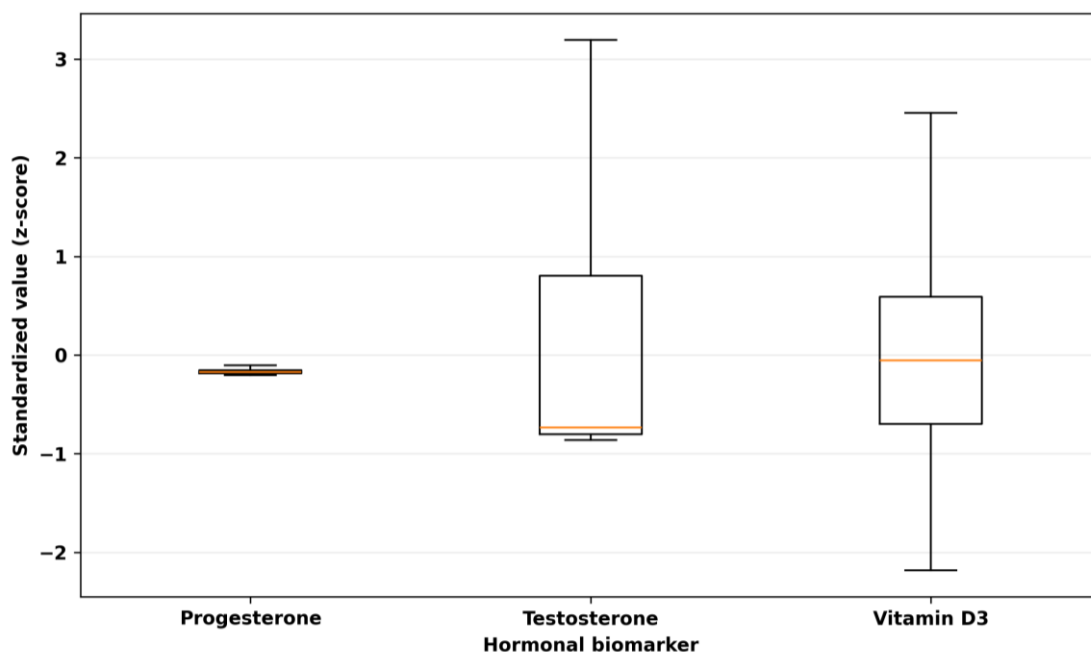


Figure 1. Distribution of hormonal biomarkers

3.2 Multivariate Dependence Between Hormonal and Immune Responses

Spearman analysis revealed that the greatest unadjusted hormone-immune connections were with testosterone (Table 2). Testosterone was positively correlated with IFN-gamma ($\rho = 0.229$, FDR $q < .001$), IL-1beta ($\rho = 0.158$, $q = .002$), IL-6 ($\rho = 0.130$, $q = .013$), and TNF-alpha ($\rho = 0.219$, $q < .001$). Progesterone was not found to have a monotonic relationship with any of the four cytokines. The vitamin D3 showed a small negative correlation with IL-6 ($\rho = -0.119$, $q = .022$). The negative correlation with IFN-gamma and TNF-alpha were not significant following false-discovery-rate

Table 2. Spearman correlations between hormonal biomarkers and LPS-stimulated immune responses

Hormone	Cytokine	N	Spearman rho	p-value	FDR q
Progesterone	IFN-gamma	484	-0.012	.792	.828
Progesterone	IL-1beta	481	-0.024	.604	.725
Progesterone	IL-6	478	-0.025	.586	.725
Progesterone	TNF-alpha	479	-0.056	.222	.334
Testosterone	IFN-gamma	484	0.229	<.001	<.001
Testosterone	IL-1beta	481	0.158	<.001	.002
Testosterone	IL-6	478	0.130	.004	.013
Testosterone	TNF-alpha	479	0.219	<.001	<.001
Vitamin D3	IFN-gamma	486	-0.087	.056	.095
Vitamin D3	IL-1beta	483	0.010	.828	.828
Vitamin D3	IL-6	480	-0.119	.009	.022
Vitamin D3	TNF-alpha	481	-0.095	.038	.075

The complete dependence pattern is visualized in Figure 2. The heat map confirms that testosterone produced the most consistently positive unadjusted correlations across the immune-response panel, whereas progesterone coefficients were concentrated close to zero.

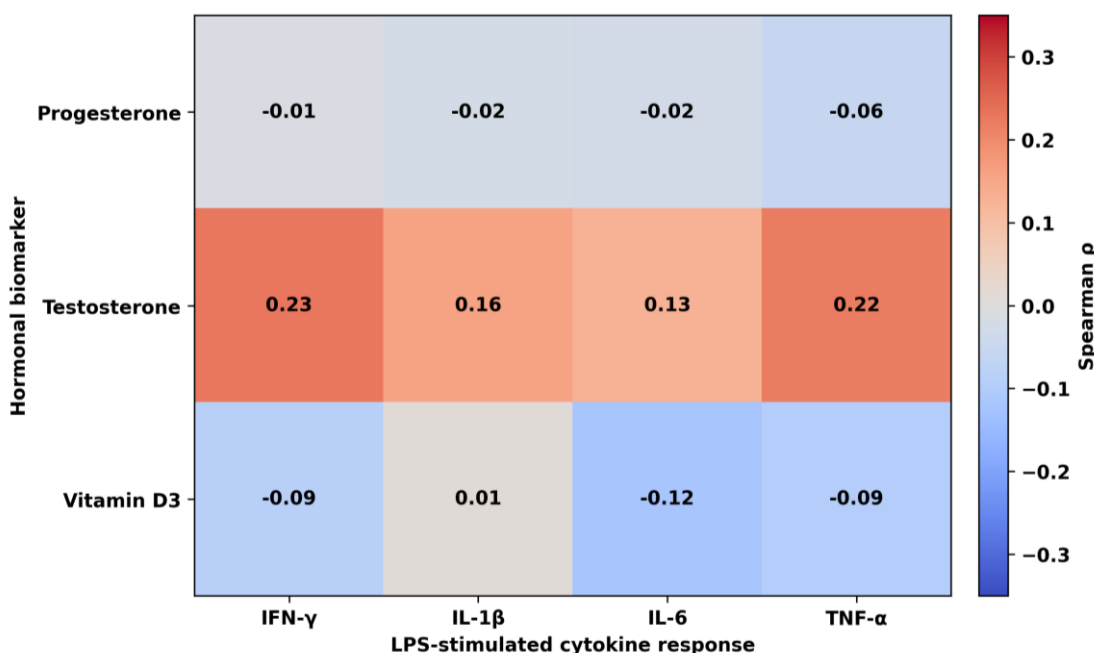


Figure 2. Hormonal-immune dependence matrix based on Spearman correlations

3.3 Adjusted Effect Estimates for Hormonal-Immune Associations

To assess the effects of physiological variation on the bivariate relationships, multivariable regression models were fit to the standardized log-transformed cytokine outcomes. Each model was adjusted for age, sex, BMI, and current smoking status and included both progesterone and testosterone and vitamin D3. Heteroskedasticity-robust standard errors were

used. All of the hormone coefficients were not statistically significant after multivariable adjustment and FDR correction as shown in Table 3. For instance, the previously positive association between testosterone and IFN-gamma was found to become negative (beta = -0.001; 95% CI: -0.204 to 0.203; q = .997), and there was an inverse but nonsignificant association between testosterone and IL-6 (beta = -0.258; 95% CI: -0.566 to 0.050; q = .524).

Table 3. Adjusted standardized associations between hormonal biomarkers and LPS cytokine responses

Cytokine outcome	Hormone	N	Standardized beta	95% CI	p-value	FDR q
IFN-gamma	Progesterone	468	0.092	-0.029 to 0.213	.135	.524
IFN-gamma	Testosterone	468	-0.001	-0.204 to 0.203	.995	.996
IFN-gamma	Vitamin D3	468	-0.028	-0.120 to 0.064	.548	.848
IL-1beta	Progesterone	465	0.030	-0.103 to 0.162	.663	.848
IL-1beta	Testosterone	465	-0.152	-0.372 to 0.068	.175	.524
IL-1beta	Vitamin D3	465	0.073	-0.017 to 0.164	.113	.524
IL-6	Progesterone	462	0.036	-0.042 to 0.114	.366	.733
IL-6	Testosterone	462	-0.258	-0.566 to 0.050	.100	.524
IL-6	Vitamin D3	462	-0.053	-0.150 to 0.044	.282	.677
TNF-alpha	Progesterone	463	0.022	-0.055 to 0.099	.578	.848
TNF-alpha	Testosterone	463	-0.001	-0.187 to 0.186	.996	.996
TNF-alpha	Vitamin D3	463	-0.019	-0.120 to 0.081	.707	.848

Note: Cytokine concentrations were $\log(1 + x)$ -transformed and standardized. Continuous hormonal predictors, age, and BMI were standardized. Models additionally controlled for sex and current smoking. Standardized beta represents the adjusted association and should not be interpreted as a causal effect.

The adjusted estimates and their 95% confidence intervals are displayed in Figure 3. All confidence intervals crossed zero,

demonstrating that the comparatively stronger bivariate testosterone associations were substantially attenuated once physiological covariates were incorporated.

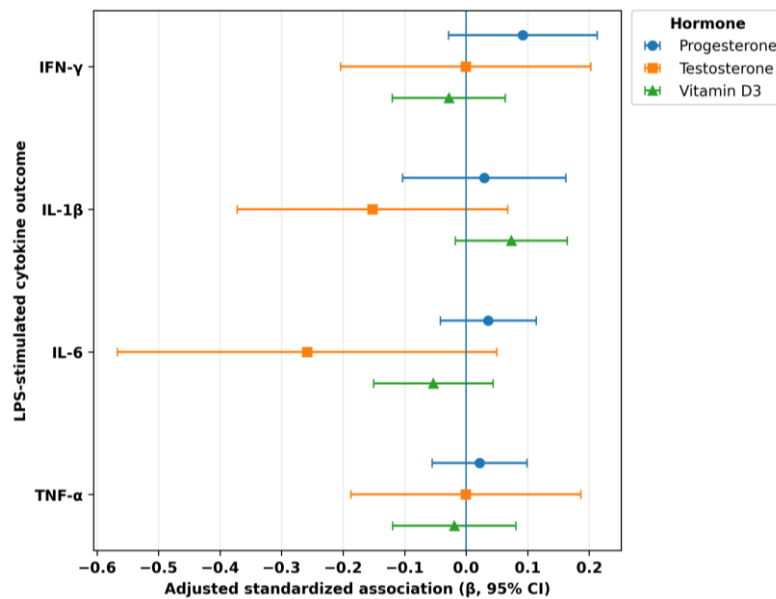


Figure 3. Adjusted standardized hormonal associations with immune responses

3.4 Physiological Variation According to Sex

Physiological variability was an important part of the data set as it included sex. As shown in Table 4, the medians for the females and males were substantially different, 0.87 and 18.60, respectively, (FDR $q < .001$). Females were more likely to have levels of Vitamin D3, with a median of 76.0 and males 64.0 ($q < .001$). There was no statistically significant difference between males and females in regards to Progesterone. Sex-related differences were also observed in all four cytokine responses to LPS. All that differed significantly between the two sexes were consistently greater in males for median responses of IFN-gamma, IL-1beta, IL-6, and TNF-alpha, with all comparisons being significant after FDR adjustment.

Table 4. Sex-related physiological variation in hormonal and immune biomarkers

Variable	Female N	Female median (Q1-Q3)	Male N	Male median (Q1-Q3)	Rank-biserial r	FDR q
Progesterone	275	0.23 (0.16-0.38)	209	0.25 (0.17-0.32)	-0.028	.596
Testosterone	275	0.87 (0.61-1.17)	209	18.60 (15.00-23.00)	-1.000	<.001
Vitamin D3	275	76.00 (57.50-97.50)	211	64.00 (44.00-78.50)	0.285	<.001
LPS IFN-gamma	278	249.50 (110.25-535.75)	211	452.00 (209.50-855.00)	-0.291	<.001
LPS IL-1beta	276	1,787.00 (1,338.75-2,299.00)	210	2,085.50 (1,633.00-2,650.25)	-0.240	<.001
LPS IL-6	276	8,736.00 (7,052.75-10,739.75)	207	9,730.00 (7,836.50-12,165.50)	-0.188	<.001
LPS TNF-alpha	277	741.00 (515.00-1,090.00)	207	1,027.00 (780.00-1,500.50)	-0.344	<.001

Note: Negative rank-biserial coefficients indicate higher values among males under the coding used in this analysis.

The Figure 4 shows the log-response values of the cytokine component of this difference in physiology due to sex. The median standardized responses were higher for males for all four cytokines, and the greatest separations were seen for IFN-gamma and TNF-alpha. This pattern also accounts for the significant reduction in the initial positive associations between testosterone and the production of cytokines, after controlling for sex and other physiological factors in the multivariable analysis.

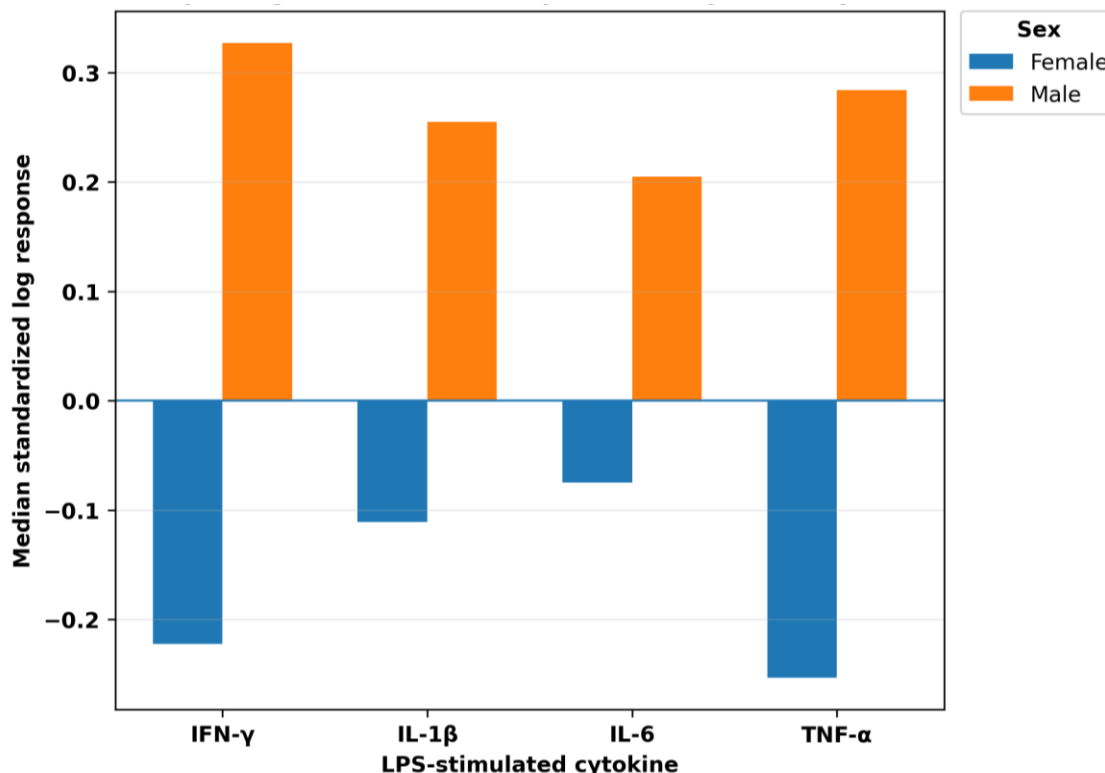


Figure 4. Physiological variation in LPS-stimulated cytokine responses according to sex

4. Discussion

The present analysis revealed considerable inter-individual differences in hormone and immune responses and showed a clear difference between the unadjusted and adjusted patterns of endocrine-immune relationships. Testosterone was correlated to all four cytokines in a bivariate fashion while progesterone was generally not monotonously associated. There was a weak inverse correlation between vitamin D3 and IL-6. None of the hormone coefficients, however, were statistically significant after simultaneous adjustment for age, sex, BMI, smoking and the other hormone variables. This attenuation suggests that this hormone-cytokine dependence was very closely linked to other physiological variation and was not just a hormonal association.

One of the most evident aspects of physiological heterogeneity was sex. Female serum blood had higher level of vitamin D3 while males' serum blood had higher level of testosterone, and males had higher level of interferon gamma, IL-1beta, IL-6, and tumor necrosis factor alpha. These results align with the emerging evidence that biological sex may influence inflammatory responses to treatment and immune activation (Canzian et al., 2025). Immune investigations in tissues have also revealed significant non-heritable variation in immune composition and activity, highlighting that immune phenotypes are not just a product of intrinsic biology, but also of the local physiological context (Bister et al., 2024). Physiological responses could be further influenced by hormonal changes throughout the female lifespan, further reinforcing the need to consider endocrine status in sex-specific analyses of biological processes (Stieger et al., 2025).

Of particular interest is the lack of important testosterone associations following multivariable adjustment. Their test population was virtually completely separated by sex, as were the concentrations of cytokines. So, physiological structure was likely a significant contributor to the unadjusted testosterone-cytokine correlations. Cowell et al., (2020) found similar sex-dependent biological effects when they investigated the relationship between maternal physiological conditions and immune-related gene expression. Comparative biology also suggests that immune function may systematically vary between the sexes, but that there are large inter-specific differences in immune function and in the mechanisms and ecological context underlying these differences (Vincze et al., 2022). More recently, there is evidence from seasonal studies of naturally occurring populations that sex-specific immunity can vary depending on the physiological and environmental state of the host, not just being fixed (Valdebenito et al., 2021).

The weak connections with progesterone further highlight that the level of circulating hormones may not be a good reflection of complex endocrine regulation. The relationships between hormones and physiology can vary by energetic condition and stress exposure, as evidenced in the studies on relation between coordinated variation in hormones, glucose and oxidative processes (Vágási et al., 2020). Immune responsiveness can also be modulated by environmental factors without affecting the measured levels of the hormones. Artificial light during exposure, for instance, has been

demonstrated to affect innate immune responses (Ziegler et al., 2021), but not to produce the same effects in more general physiological measures as population density (Kamiński et al., 2022).

The inverse association between vitamin D3 and IL-6 that was observed, however, did not result in an adjusted coefficient. This implies that the immune variation described here is of vitamin D and should not be considered as a direct independent effect. In general, immune systems are affected by many environmental and physiological changes. It has been reported that spaceflight induces changes in immune function due to both environmental and physiological stresses (Akiyama et al., 2020). Variation in glucocorticoid levels also captures complex exposure to environmental and anthropogenic stressors among free-ranging mammals (Pirotta et al., 2023). The effect of photoperiod history can also result in changes of immune responses and infection susceptibility (Whiting et al., 2020).

Last, the large inter-individual variability of cytokine levels within each stimulation distribution suggests significant inter-individual differences in immunity even for the same stimulation protocol. This heterogeneity may have biological significance as there is evidence of fitness-related differences in innate immune system variation in experimental populations (Roast et al., 2020).

Overall, the results confirm the use of a multivariate combined phenotype approach for analyzing the hormonal and immune parameters. They highlight that apparent endocrine-immune links may vary greatly once physiological covariates are considered and therefore stress the need to adjust for such factors when interpreting hormonal correlates of immune responsiveness.

5. Conclusion

This study showed that there is significant inter-individual variability in the hormonal and immune response and that these must be analysed in a multivariate framework of physiology. Testosterone was positively associated with LPS stimulated levels of interferon gamma, IL-1beta, IL-6, and tumor necrosis factor alpha with little correlation found with progesterone levels. There was a slight inverse correlation between vitamin D3 and IL-6. These hormone-immune associations, however, did not hold after the mutual adjustments for age, sex, BMI, smoking, and the other hormonal variables, suggesting that such dependence structure was significantly modified by the other physiological associations considered. The variation between sexes was marked. The testosterone levels were significantly higher in males, and the cytokine responses were significantly stronger, while vitamin D3 levels were higher in the females. These differences highlight the importance of considering sex as a major source of biological heterogeneity when evaluating endocrine-immune relationships. In general, the results suggest that the apparent relationship between hormonal biomarkers and immune responsiveness may be due to underlying physiological variation to some extent, rather than to independent effects of the hormones. The analysis of two or more variables (multivariable modelling) is therefore more reliable in terms of interpretation than the analysis of two variables only (bivariate analysis). Longitudinal measurements repeated hormonal evaluations, more comprehensive inflammatory panel, and other behavioral and metabolic measurements should be considered for future studies to establish temporal and perhaps causal relationships in endocrine-immune regulation.

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