Changes in Proteome Profiles Linked to Hormonal Contraceptive Use Among African American Women with untreated high blood pressure

Lisa DeRoo[1], Malak Abbas[1], Gabriel Goodney[1], Amadou Gaye[1*]

[1]National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA
* Corresponding author: amadou.gaye@nih.gov, 10 Center Drive, Room 7N316, Bethesda, MD 20892

ABSTRACT

Background

The use of oral contraceptives among women with pre-existing cardiometabolic risk factors has been associated with increased risk of cardiovascular events but the underlying mechanisms are not well understood. Analyzing the proteome enables the identification and quantification of proteins involved in various cellular processes, including those affected by oral contraceptives.

Objective

The objective of this study was to investigate if current oral contraceptive use was associated with alterations in the plasma proteome among pre-menopausal African American women with hypertension in the Genomics, Environmental Factors, and the Social Determinants of Cardiovascular Disease in African Americans Study (GENE-FORECAST).

Study Design

We evaluated 2,941 serum proteins measured on the Olink platform to assess the associations between oral contraception use and protein levels among 51 pre-menopausal women with untreated Stage 2 hypertension (13 oral contraception users and 38 non-users). A generalized linear model was fitted for each protein, adjusting for age, body mass index, alcohol consumption, level of education, and physical activity. Pathway enrichment analysis was carried out to identify common pathways among the proteins associated with oral contraceptive use. Gene Ontology enrichment analysis was conducted to gain insight into the functional characteristics of the proteins and the underlying biology.

Results

We identified 44 proteins significantly associated with oral contraceptive use. Among those, 31 showed decreased levels among oral contraceptive users, while 13 exhibited an increase in users. Fourteen proteins were enriched in potentially relevant pathways for the pathogenesis of cardiovascular disease including complement and coagulation cascades, cholesterol metabolism and Notch signaling. There were 25 enriched Gene Ontology terms encompassing 39 of the 44 proteins linked to oral contraceptive use; several of these are involved in peptidase inhibitor activity.

Conclusions

This study provides novel insights into the proteomic landscape associated with oral contraceptive use among African American women with uncontrolled hypertension.

Key words

cardiovascular risk, women's health, oral contraception, proteome, hypertension, African Americans

INTRODUCTION

Hypertension is a main contributor to cardiovascular disease (CVD), the major cause of death worldwide.[1] Sex is one of the most important modulators of CVD risk and response to treatment, and understanding sex-specific differences in the pathophysiology of CVD is a necessary step for personalized medicine.[2] Female-specific risk factors are understudied in basic, clinical and population research, with perhaps overlooked opportunities for prevention in younger women.[3] Hypertension is a health concern for women of reproductive age because it complicates pregnancies and impairs quality of life.[4] In a nationally representative 2011-2016 survey conducted in of non-pregnant women of reproductive age, 9.3% had hypertension; among those, 16.9% were unaware that they had hypertension and over 40% had uncontrolled hypertension.[5] Cumulative exposure to elevated blood pressure (BP) over time is associated with increased risk of CVD outcomes and mortality, above and beyond current BP status. This suggests the importance of a life course approach to hypertension, with accumulated years of elevated BP in early to mid-adulthood having longer term implications for CVD risk.[6] There are significant racial disparities for African Americans compared to U.S. whites in the prevalence, severity and control of hypertension. African American women have a higher age-adjusted prevalence of hypertension (56.7%) than non-Hispanic white women (36.7%)[7] and tend to develop hypertension at earlier stages of life and endure more severe manifestations of the condition.[8] Among younger, premenopausal women with hypertension, African Americans were over two times more likely to have uncontrolled BP than whites.[9]

Oral contraceptive pills (OCs) are widely used for the prevention of pregnancy, and are also prescribed for alleviating symptoms of menstrual disorders, endometriosis, and polycystic ovarian syndrome.[10] OCs have also been linked with an increased risk of cardiovascular events such as venous thromboembolism, myocardial infarction, and ischemic and hemorrhagic stroke,[10] although these risks have been mitigated over the successive generations of OCs developed to reduce side effects.[11] The most commonly used OCs today are combined pills containing lower doses (<50 ug) of ethinyl estradiol (EE) and different formulations of progestin than earlier generations of pills. [12, 13] Even low dose combined OCs are associated with a small, reversible increase in BP due to the estrogen component. Among healthy, reproductive-aged women with a low absolute risk for CVD, the small increase in BP due to OC use is unlikely to be clinically relevant. However, for women with multiple risk factors (age > 35, smoker, high blood pressure), OC-use may increase CVD risk to an unacceptable level. The mechanisms for how OCs increase BP are not fully understood. Hypotheses include estrogen-induced activation of the renin-angiotensin-aldosterone system (RAAS), impaired RAAS feedback inhibition, increased sympathetic nervous system activity, and altered regulation of antidiuretic hormone (ADH).[10] Given that CVD risk increases with cumulative exposure to

hypertension and that African Americans have greater prevalence and earlier onset of hypertension, understanding how OC-use may contribute to or exacerbate hypertension is of particular importance in African American women.

In this project, we investigated the proteome profiles of pre-menopausal, hypertensive African American women to uncover differences in plasma proteins linked to OC use. Proteomics provides insights into real-time human biology by specifying the protein profile at the time of sampling; the comparison and relative quantification of proteins under a defined set of conditions can help identify relevant proteins linked to particular exposures or disease states.[14] We aimed to identify and quantify plasma proteins involved in cellular processes related to OC use by examining associations between proteins and OC use. The motivation behind this research is to enhance the understanding of how OC use contributes to the pathogenesis of CVD among women with existing hypertension, potentially forming a framework for the development of individualized strategies for contraception among this high risk group. There have been only a few studies examining OC use and the plasma proteome, and they examined between 54 and 171 proteins.[15-17] Here we conducted an unbiased analysis of 2,941 serum proteins to allow for the discovery of novel OC-related proteins and provide for a more comprehensive exploration of the proteome.

MATERIAL AND METHODS

Study population

We used data from the GENomics, Environmental FactORs, and the Social DEterminants of Cardiovascular Disease in African Americans STudy (GENE-FORECAST), a study of 669 self-identified, U.S.-born African American men and women aged 21 to 65 years, recruited from the metropolitan Washington D.C. area during 2014-2022. The study protocol was approved by the Institutional Review Board of the U.S. National Institutes of Health (NIH), and written informed consent was provided by all participants. Data collection took place during a visit to the NIH Clinical Research Center. Pregnancy was an exclusion criterion for the study, and women were asked about menopausal status before undergoing a mandatory pregnancy test. Participants provided blood samples, underwent a physical exam and filled out questionnaires on medical history and health behaviors. BP measurements were taken in triplicate and averaged. OC use was obtained in a questionnaire aimed at collecting a comprehensive list of current medications.

GENE-FORECAST employs a multi-omics systems biology approach, facilitating comprehensive, multi-dimensional characterization of health and disease among African Americans. Here, we aimed to isolate the differences in protein profiles for OC use among African American women with uncontrolled hypertension. Our inclusion criteria were women of pre-menopausal status with stage 2

hypertension (defined as systolic BP >=140 mm HG or diastolic BP >=90 mm HG based on the average of clinic measurements) who were not taking anti-hypertensive medication.

Proteomic data

The ethylenediamine tetraacetic acid (EDTA) serum samples from the GENE-FORECAST were sent to the Olink Proteomics Analysis Service in Boston, USA. Proteomic analyses were conducted collectively in a single batch. The Explore 3072 assay, utilizing eight Explore 384 Olink panels (Cardiometabolic, Cardiometabolic II, Inflammation, Inflammation II, Neurology, Neurology II, Oncology, Oncology II), was run to assess the relative expression of a total of 2,947 proteins. Proximity Extension Assay (PEA) technology was conducted according to the Olink AB manufacturer procedures by the certified laboratory. Briefly, the technique relies on the use of antibodies labelled with unique DNA oligonucleotides that bind to their target protein present in the sample. The DNA oligonucleotides, when in proximity on a target protein, undergo hybridization and act as a template for DNA polymerase-dependent extension, forming a unique double-stranded DNA barcode proportionate to the initial protein concentration. Quantification of resulting DNA amplicons is accomplished through high-throughput DNA sequencing, generating a digital signal reflective of the number of DNA hybridization events corresponding to the protein concentration in the original sample. The measurement of protein levels is based on Normalized Protein eXpression (NPX) values, serving as a relative protein quantification unit. This quantification is normalized to account for systematic noise arising from sample processing and technical variation, leveraging internal controls and sample controls. NPX units are on a log2 scale, where a one NPX unit increase indicates a two-fold rise in the concentration of amplicons representing the target protein compared to the internal control. A total of 2,941 proteins that passed quality controls were were included in the analysis.

Statistical analysis

Separate generalized linear models were fit for each of the 2,941 proteins in which the protein level was the outcome variable and the main exposure of interest was oral contraceptive use (no=0; yes=1), adjusting for age, body mass index (BMI), physical activity level, and education level. The p-values of the associations were adjusted for false discovery rate (FDR) using the Benjamini & Hochberg (BH) approach, with an FDR adjusted p-value ≤ 0.05 considered statistically significant.

Pathway enrichment analysis was undertaken to identify molecular pathways enriched in the set of proteins found to be associated with OC use. This analysis was carried out with the R algorithm *pathfinR* ,[18] a tool that defines subnetworks in a cluster of genes and identifies pathways over-represented in those subnetworks. Pathways with a BH adjusted enrichment p-value ≤ 0.05 were considered significantly overrepresented.

Gene Ontology (GO) enrichment analyses was subsequently conducted with the set of proteins found to be associated with OC use to provide a systematic framework for annotating and deciphering biological functions, processes, and cellular components linked with the proteins and to gain insight into their roles within cellular and organismal contexts. GO analysis were conducted using the R libraries 'enrichGO' which runs a hypergeometric test to determine whether the set of proteins associated with OCs is enriched for specific GO terms compared to what would be expected by chance. GO terms with a BH adjusted enrichment p-value ≤ 0.05 were considered significantly overrepresented.

RESULTS

Our study sample included 51 pre-menopausal women with stage 2 hypertension; 13 OC users and 38 non-users (Table 1). OC users and non-users were similar in regard to mean age, 37 and 38 years, respectively and mean BP measurements (181-180 mm HG Systolic BP and 72 mm HG Diastolic BP). Nearly all subjects were non-smokers. OC users were less likely to have a healthy BMI and were more likely to drink alcohol, be a college graduate, and engage in moderate or greater than moderate physical activity than non-users.

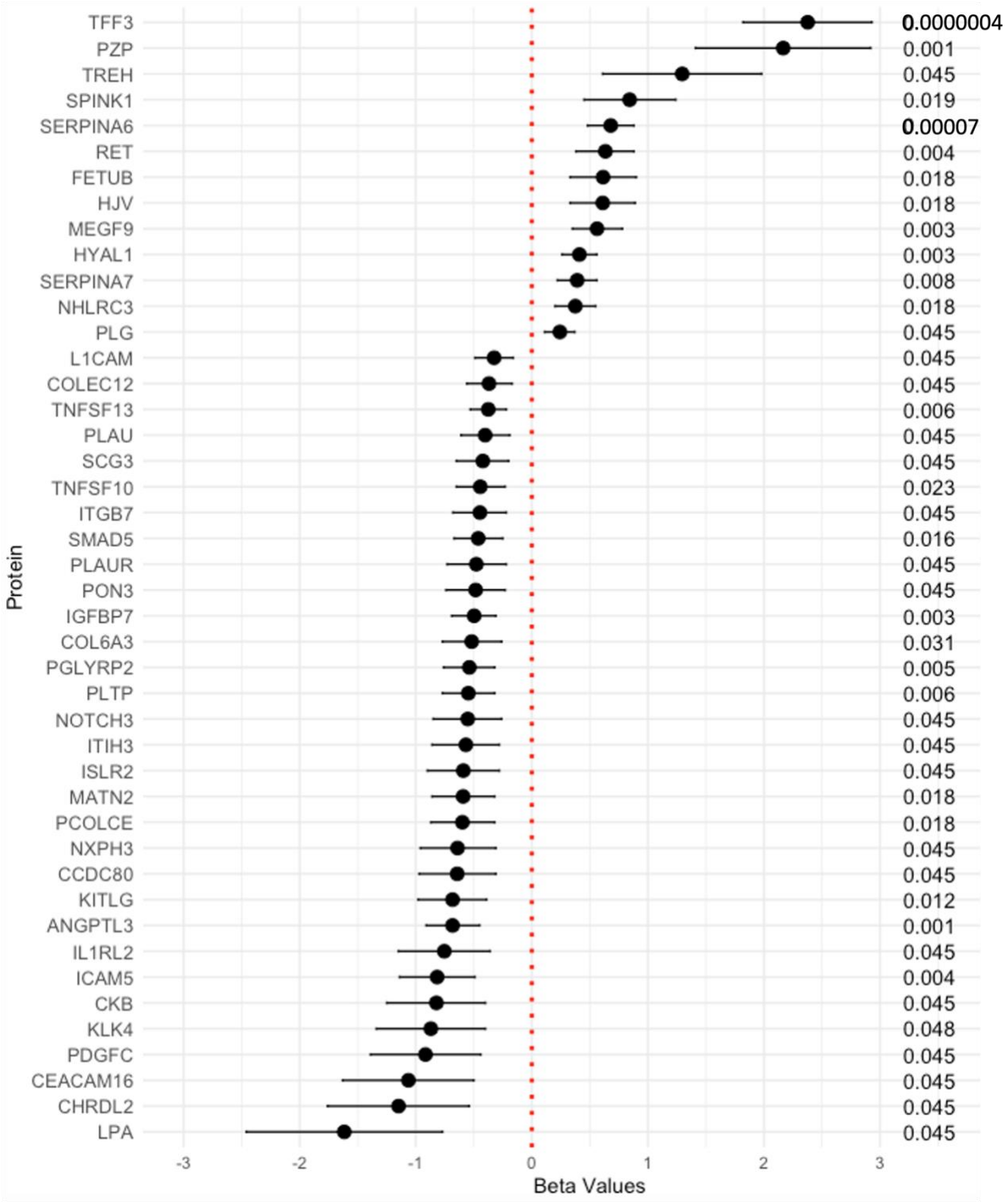
Table 1: Baseline characteristics of the 53 women included in the analysis.

Characteristics	**No HC (n = 38)** Mean / Count (SD / %)	**HC (n = 13)** Mean / Count (SD / %)
Age (years)	38 (9)	37 (10)
Systolic Blood Pressure (mmHg)	180 (23)	181 (20)
Diastolic Blood Pressure (mmHg)	72 (10)	72 (13)
Body Mass Index (kg/m^2)	31 (8)	32 (6)
Current Smoker		
No	36 (95%)	13 (100%)
Yes	1 (3%)	0 (0%)
Drinks Alcohol		
No	12 (32%)	2 (15%)
Yes	25 (66%)	11 (85%)
Education Level		
≤ High School	11 (29%)	2 (15%)
High School + Vocational Educational	13 (34%)	4 (31%)
≥ Graduate Degree	12 (32%)	7 (54%)
Physical Activity (Baeckes' sport index)		
< Moderate Activity	17 (45%)	5 (38%)
≥ Moderate Activity	21 (55%)	8 (62%)

Proteins Associated with OC Use

We identified 44 proteins significantly associated with oral contraceptive use (Figure 1 and Supplemental Table T1). There were 31 proteins exhibiting lower levels among OC users and 13 proteins with higher levels.

Figure 1: Summary proteins linked to hormonal contraceptive use, including beta coefficients with their corresponding 95% confidence intervals and adjusted p-values.



Pathway Enrichment Analysis

Among the 44 proteins associated with OC use, pathway enrichment analysis identified 14 proteins enriched in relevant pathways like TGF-beta and Notch signaling (Table 3). Among these 14 proteins, two exhibited elevated levels in OC users, while 12 displayed decreased levels.

Table 2: Pathways overrepresented in the set 44 proteins associated with hormonal contraceptive use. The last two columns indicate the protein involved in the pathway and the direction of the change in the HC users group.

Pathway ID	Description	adjusted P-Value	Up regulated	Down regulated
hsa04610	Complement and coagulation cascades	0.00002	PLG	PLAU, PLAUR
hsa05215	Prostate cancer	0.00024		PLAU, PDGFC
hsa05205	Proteoglycans in cancer	0.0023		PLAU, PLAUR
hsa04979	Cholesterol metabolism	0.0044		PLTP, LPA, ANGPTL3
hsa05216	Thyroid cancer	0.0083	RET	
hsa03320	PPAR signaling pathway	0.0098		PLTP
hsa04330	Notch signaling pathway	0.012		NOTCH3
hsa05230	Central carbon metabolism in cancer	0.029	RET	
hsa04350	TGF-beta signaling pathway	0.030		SMAD5
hsa04658	Th1 and Th2 cell differentiation	0.031		NOTCH3
hsa05223	Non-small cell lung cancer	0.032	RET	
hsa05218	Melanoma	0.032		PDGFC
hsa04371	Apelin signaling pathway	0.034		NOTCH3
hsa04514	Cell adhesion molecules	0.041		ITGB7, L1CAM
hsa04540	Gap junction	0.045		PDGFC
hsa04512	ECM-receptor interaction	0.047		COL6A3, ITGB7

Gene Ontology Enrichment Analysis

GO enrichment analysis revealed 25 enriched GO terms involving 39 of the 44 proteins (Table 4). Several proteins were implicated in peptidase inhibitor activity, including members of the serpin family.

Table 3: GO terms overrepresented in the set 44 proteins associated with hormanal contraceptive use.

GO ID	Description	adjusted P-Value	Proteins
GO:0061134	peptidase regulator activity	0.0000003	SERPINA6, PZP, SERPINA7, FETUB, PCOLCE, SPINK1, COL6A3, ITIH3, LPA
GO:0004866	endopeptidase inhibitor activity	0.0000003	SERPINA6, PZP, SERPINA7, FETUB, SPINK1, COL6A3, ITIH3, LPA
GO:0030414	peptidase inhibitor activity	0.0000003	SERPINA6, PZP, SERPINA7, FETUB, SPINK1, COL6A3, ITIH3, LPA
GO:0061135	endopeptidase regulator activity	0.0000003	SERPINA6, PZP, SERPINA7, FETUB, SPINK1, COL6A3, ITIH3, LPA
GO:0004867	serine-type endopeptidase inhibitor activity	0.0000031	SERPINA6, PZP, SERPINA7, SPINK1, COL6A3, ITIH3

GO:0004857	enzyme inhibitor activity	0.0000048	SERPINA6, ANGPTL3, PZP, SERPINA7, FETUB, SPINK1, COL6A3, ITIH3, LPA
GO:0005539	glycosaminoglycan binding	0.0041	ANGPTL3, PGLYRP2, PCOLCE, CCDC80, LPA
GO:0005201	extracellular matrix structural constituent	0.0072	IGFBP7, MATN2, PCOLCE, COL6A3
GO:0030169	low-density lipoprotein particle binding	0.0072	PLTP, COLEC12
GO:0034185	apolipoprotein binding	0.0072	LPA, PLG
GO:0004252	serine-type endopeptidase activity	0.0072	LPA, PLAU, PLG, KLK4
GO:0008201	heparin binding	0.0072	ANGPTL3, PCOLCE, CCDC80, LPA
GO:0036122	BMP binding	0.0089	HJV, CHRDL2
GO:0008236	serine-type peptidase activity	0.0089	LPA, PLAU, PLG, KLK4
GO:0017171	serine hydrolase activity	0.0090	LPA, PLAU, PLG, KLK4
GO:0001968	fibronectin binding	0.015	CCDC80, LPA
GO:0005164	tumor necrosis factor receptor binding	0.015	TNFSF13, TNFSF10
GO:0071813	lipoprotein particle binding	0.015	PLTP, COLEC12
GO:0071814	protein-lipid complex binding	0.015	PLTP, COLEC12
GO:0038187	pattern recognition receptor activity	0.017	PGLYRP2, COLEC12
GO:0016798	hydrolase activity, acting on glycosyl bonds	0.020	HYAL1, IL1RL2, TREH
GO:1901681	sulfur compound binding	0.021	ANGPTL3, PCOLCE, CCDC80, LPA
GO:0005178	integrin binding	0.029	ANGPTL3, ICAM5, ITGB7
GO:0032813	tumor necrosis factor receptor superfamily binding	0.029	TNFSF13, TNFSF10
GO:0008083	growth factor activity	0.030	ANGPTL3, KITLG, PDGFC

DISCUSSIONS

Principal Findings

In our proteomic study of 51 pre-menopausal African American women with stage 2 hypertension (13 OC users and 38 non-users), we identified 44 proteins significantly associated with OC use. Eight of these proteins have been identified in previous studies, with the remaining, to our knowledge, not previously known to be associated with OC use. Our findings support previously described hypothesized mechanisms for the effect of OC use on hypertension, including the activation of RAAS, impaired RAAS feedback inhibition, increased sympathetic nervous system activity, and altered regulation of antidiuretic hormone (ADH).[10] We found significant differences in the levels of SERPINA6 (corticosteroid-binding globulin) and SERPINA7 (thyroxine-binding globulin) among OC users, supporting the RAAS activation hypothesis. These proteins play crucial roles in hormone transport and may indirectly influence RAAS components, leading to increased sodium and water retention and elevated BP. TNFSF13 and TNFSF10, involved in chronic inflammation, aligns with the impaired RAAS feedback inhibition hypothesis. Chronic inflammation could impair the feedback mechanisms that regulate RAAS, exacerbating hypertension in OC users. Increases in SCG3

(secretogranin III), PLAU (plasminogen activator urokinase), and PLAUR (plasminogen activator urokinase receptor) levels support the hypothesis that OCs enhance sympathetic nervous system activity, contributing to elevated BP. These proteins are involved in neuroendocrine secretion, tissue remodeling, and inflammation, potentially affecting sympathetic activity. Lastly, the observed changes in LPA (lysophosphatidic acid) and COL6A3 (collagen type VI alpha 3 chain) levels suggest that OCs may alter ADH regulation, influencing cell signaling, fluid balance, and extracellular matrix structure, which are critical for maintaining BP homeostasis. These findings collectively support the notion that hormonal contraceptives may contribute to hypertension through multiple interconnected molecular pathways.

Results in the Context of What is Known

The following eight proteins overlapped with the results of previous studies: PZP, SERPINA6, FETUB, NHLRC3, PLG, PGLYRP2, and ITIH3 identified in the CHRIS Study conducted in South Tyrol, Italy,[17] and TFF3 identified in the Netherlands Study of Depression and Anxiety.[16] Reassuringly, our associations show the same directionality and similar magnitude of effect as the previous findings. Mechanistically, these proteins play pivotal roles in modulating vascular and inflammatory responses. Upregulation of TFF3, known for mucosal protection and repair,[19] suggests a potential response to OC-induced vascular endothelial damage. FETUB is part of the PI3K/AKT signaling pathway, disruptions in which can lead to endothelial dysfunction, a precursor to CVD, particularly in altered hormonal environments.[20] ITIH3 has been linked to myocardial infarction, with a functional SNP enhancing its transcriptional level, underscoring its significant influence on vascular integrity and inflammatory processes.[21] Protease inhibitors PZP and PGLYRP2 promote vascular inflammation and endothelial dysfunction, crucial in the development of hypertension.[22] Together, these proteins demonstrate the complex interplay between OC use and proteomic changes, offering insights into the pathophysiological processes that may contribute to known cardiovascular risks.

We also identified proteins that, to our knowledge, have not been previously linked to OC use. For example, SMAD5, a central signaling molecule in the TGF-beta pathway is known for its influence on vascular remodeling and fibrosis.[23] SMAD5's role in maintaining blood vessel integrity implies its impact on BP regulation and hypertension pathogenesis.[24] OC use may alter TGF-beta signaling via changes in SMAD5 levels, potentially affecting hypertension-associated vascular changes. [25] ANGPTL3, a key player in regulating lipid metabolism,[26] was decreased in OC users. ANGPTL3 typically modulates lipoprotein lipase, and its reduced levels might result in lower triglyceride levels, usually seen as protective against CVD.[27] However, in the context of hypertension, the effects of altering lipid metabolism are nuanced and may not uniformly benefit cardiovascular health. The

decrease of ANGPTL3 could signify alterations in the lipid profile of OC users, potentially exacerbating atherosclerosis associated with hypertension.[28]

Pathway Analysis

Pathway analysis revealed enrichment in complement and coagulation cascades, with notable changes in PLG, PLAU, and PLAUR expression. These proteins are associated with inflammatory responses and blood coagulability,[29, 30] suggesting an OC-induced prothrombotic state that increases the risk of cardiovascular events in hypertensive women.[31] Changes in proteins related to cholesterol metabolism, such as PLTP, LPA, and ANGPTL3, underscore the potential impact of OCs on lipid levels and, consequently, cardiovascular health.[32] The modulation of the Notch signaling pathway, particularly through NOTCH3 also indicates potential alterations in vascular remodeling and arterial pressure regulation.[33] While the enriched pathways encompass a range of functions, including some cancer-related pathways, these pathways often involve genes fundamental to cellular processes like cell cycle regulation, DNA repair, and apoptosis. Consequently, genes associated with cancer pathways may participate in diverse biological processes beyond cancer biology.

Insights from GO Analysis

The GO analysis reveals overrepresented of pathways that encompass proteins involved in peptidase inhibitor activity, including members of the serpin family such as SERPINA6 and SERPINA7.[34] These proteins regulate protease activity, critical for maintaining vascular integrity and BP homeostasis.[35] By influencing the proteolytic balance, OCs may affect the vascular response to hypertension. Growth factor activity-related proteins like PDGFC and KITLG suggests potential effects on endothelial function and vascular tone, affecting BP control.[36] Growth factors are pivotal in vascular repair and homeostasis,[37] indicating that OCs may exert effects on endothelial function and vascular tone, with consequential implications for BP regulation.

Clinical Implications

Our investigation helps lay the framework for CVD-related biomarker discovery in OC users with hypertension and contributes to the scientific knowledge of how OC use may affect biological processes leading to CVD. For women with hypertension or other CVD risk factors, tailored, patient-centered contraceptive counseling is important to mitigate the potential adverse effects elucidated by our proteomic and pathway analyses. Future work in the proteomics of OCs may lead to a personalized medicine approach to contraception, creating tailored contraceptives that best match a woman's needs for efficacy and health.[38]

Research Implications

We have replicated previous proteomic findings regarding OC use and have identified previously unknown proteins associated with OC use. Research is needed to understand in more detail the proteins that are linked to the pathogenesis of CVD linked to OC use.

Strengths and limitations

Our study used a robust proteomic approach to investigate changes in protein expression associated with OC use and examined nearly 3,000 proteins, the largest number to date in proteomic studies of OC use. Our incorporation of pathway and gene ontology analyses adds depth to the interpretation of proteomic data, revealing enriched biological pathways and molecular functions, thereby enhancing the understanding of underlying mechanisms.

The GENE-FORECAST Study did not record detailed information on OCs such as the formulation, dosage and duration of use. Therefore, we could not evaluate the effects of these OC characteristics on the proteome. Most of our exposed women were probably taking combined OCs containing a synthetic estrogen component and progestin, the most common type of OC used in the United States. The use of progestin-only pills, the other category of OCs, is very low in the United States, making up about 4% of all OC users.[39] We also lacked information on the use of non-pill forms of hormonal contraceptives such as the combined estrogen/progestin transdermal patch and vaginal ring. To the extent that any of our OC non-users were using other forms of hormonal contraception, the resulting misclassification would have made it harder to detect proteomic differences between the groups. The cross-sectional nature of the analysis limits the ability to establish causality between OC use and proteomic alterations, as temporality cannot be inferred from the data. The study subjects were African American women, and results may not be generalizable to other populations with different demographic characteristics. However, as noted above, 8 of the 44 proteins associated with OC use in our study were previously identified in European populations.[16, 17] Despite adjusting for several confounding variables in the regression analysis, there may be residual confounding due to unmeasured factors such as dietary habits, which may have influenced the observed associations. Finally, we acknowledge that our findings require validation in independent cohorts to confirm their reproducibility and generalizability.

Conclusions

OCs exert influence on a spectrum of proteins implicated in vital pathways governing coagulation, lipid metabolism, and endothelial function, all pivotal for the cardiovascular well-being of hypertensive individuals. The intricate web of connections between the modified proteins and the enriched pathways delineates a network of biological processes potentially modulated by OCs. This

network represents a multifactorial framework for the increased CVD risk associated with OC use in the context of hypertension. This analysis enhances our understanding of OC use among women with hypertension, potentially informing individualized risk assessment and management strategies.

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