Sex specific vitamin D effects on blood coagulation among overweight adults

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Abstract

Background: Overweight adults are at increased risk for cardiovascular disease and vitamin D deficiency, whereas an important feature to vitamin D physiology is its sex dependence. The aim of this study was to examine whether vitamin D status improvement exerts a sexually dimorphic effect on serum proteins associated with cardiovascular risk among overweight adults.

Methods: Unprocessed serum from age- and BMI-matched men (n=26) and pre-menopausal women (n=24) with vitamin D deficiency and after they achieved sufficiency through a 12-month nutritional intervention was analyzed using our previously published depletion-free, quantitative proteomics method. Key findings were verified with ELISA. Differentially expressed proteins were subjected to *in silico* bioinformatics assessment using Principal Component Analysis, hierarchical clustering and Metacore™ pathway analysis. All mass spectrometry proteomic data are available via

ProteomeXchange (identifier: PXD003663).

Results: A total of 282 proteins were differentially expressed after the intervention between men and women (*p-value ≤ 0.05*), in which the blood coagulation pathway was significantly enriched. In agreement with the proteomics findings, ELISA measurements showed vitamin-K dependent protein C, von Willebrand factor, fibrinogen gamma chain and multimerin-1 proteins, of relevance to blood coagulation, to be differentially affected (*p-value ≤ 0.05*) between sexes after vitamin D status correction.

Conclusions: This study identified novel protein-level molecular indicators on the sexually dimorphic effect of vitamin D status correction associated with blood coagulation among overweight adults. These sex-mediated vitamin D effects should be factored in the design and interpretation of vitamin D observational and interventional studies testing cardiometabolic outcomes.

Introduction

Vitamin D deficiency is essentially pandemic, with an estimated one billion people affected worldwide [1]. Apart from its well-established role in calcium and phosphorus homeostasis, vitamin D deficiency has been implicated with adverse extra-skeletal health outcomes, including cardiovascular disease (CVD) [2]. Based on the results of a recent umbrella review, there is suggestive evidence for an association between increased serum vitamin D levels and lower risk of type II diabetes mellitus, hypertension and cardiovascular disease [3]. NHANES III, a cross-sectional study, found an adjusted hazard ratio for CVD mortality of 2.36 (95% confidence intervals 1.17 to 4.75) after seven years of follow-up, for subjects with 25(OH)D levels below 25 nmol/L compared to those with levels over 100 nmol/L [4]. One more observational study that followed-up 10,000 adults 50 to 74 years old for approximately seven years, showed that subjects with vitamin D deficiency had a 27% increased risk of total CVD as well as a 62% increased risk for fatal CVD [5].

Adults with overweight or obesity are at increased risk for both vitamin D deficiency [6] and CVD [7]. A recent systematic review by the authors highlighted a paucity of vitamin D interventional studies with cardiometabolic risk factors as outcomes among otherwise healthy overweight and obese adults [8]. Within this context, whether vitamin D status improvement, as part of an interventional protocol, exerts sexual dimorphism in pathways associated with cardiovascular disease at the serum protein level has not been examined to date. Recent studies, including the authors', have demonstrated that vitamin D disposition manifests marked sexual dimorphism, also evident through its effects on sex hormone metabolism [9-12].

Recent notable reviews report on the increasing utility of proteomics to various aspects of nutrition research [13, 14]. In particular, non-targeted, global proteomics could be used to identify novel targets of response to a nutritional intervention at the tissue or serum/plasma level, thus forming the basis to a "hypothesis-generating" strategy [15]. Also, proteomics could be used for the "hypothesis-driven" analysis of *a priori* selected proteins. A targeted proteomics pipeline has been used in a recent vitamin D observational study examining how vitamin D status differentially affects the expression of 54 high-abundant plasma proteins between hormonal contraceptive users and non-users [16]. To date, no studies have reported the non-targeted quantitative analysis of serum proteins that become altered consequent to a vitamin D intervention protocol. This is, in part, consequent to the technically challenging task of effectively analysing the serum proteome to elucidate clinically relevant protein markers [17].

The principle aim of this study was to compare the effects of vitamin D status correction on serum proteins associated with cardiovascular risk, between age- and BMI-matched overweight males and pre-menopausal females using the author's in-depth proteome analysis of non-depleted whole serum [10, 18, 19].

Materials and Methods

Recruitment of participants and intervention

Reporting of this study conforms to the STROBE statement and the broader EQUATOR guidelines [20]. The authors [21] previously reported the study design and ethics approval by the King Saud University (KSU) Ethics Committee. To adjust for possible seasonal effects on baseline 25(OH)D serum levels, participants were recruited within the months of December and January through advertising in four Primary Health Care Centres (PHCC) of the Riyadh Central Region. A total of 50 (24 female; 26 male) adults (20 to 35 years old) with overweight (BMI from 25 to 30 kg/m^2) but otherwise healthy except for vitamin D deficiency [25(OH)D levels < 50 nmol/L] were included (**Table 1**). This threshold of serum 25(OH)D levels was selected in accordance with the definition of vitamin D deficiency by the Institute of Medicine (IOM) [22]. Patients with obesity, morbid obesity, type 1 or type 2 diabetes, smokers, pregnant or breastfeeding women, post-menopausal women, and subjects taking vitamin or botanical (i.e. St. John's Wort, Ginkgo biloba, Echinacea, etc.) supplements, NSAIDS, aspirin or any other non-prescription medicinal product were excluded from the study. All participants signed an informed consent form. Subjects were asked to return after an overnight fasting for anthropometry and blood withdrawal. This procedure was repeated at 12 months. During the 12-month intervention, subjects were advised to expose themselves to sunlight before 12 am or after 3 pm for 5 to 30 min daily and also encouraged through weekly SMS to increase their daily consumption of vitamin D-rich foods (vitamin D-fortified fresh milk, yoghurt and cheese). Serum levels of 25(OH)D were used as a reliable surrogate marker of vitamin D repletion [25(OH)D levels > 50 nmol/L] [23]. Compliance to the advice for increased sun exposure was assessed through diaries the participants kept for the whole duration of the intervention and questionnaires at baseline and after the end of the intervention. Total energy, macronutrient (carbohydrate, fibre, protein, fat) and micronutrient [saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), vitamin D, calcium] intake were estimated at baseline and after the end of the study using food frequency questionnaires (**Supplementary section 1**). Physical activity levels of the participants were assessed at baseline and after the end of

the study in compliance with the Global Physical Activity Questionnaire developed by the WHO [24] (**Supplementary section 2**). All participants completed the 12-month intervention.

Anthropometry and blood collection

Participants were asked to visit their local PHCC physician and/or nurse on duty after overnight fasting (>10 hours) for anthropometry and blood withdrawal. Anthropometry included height (rounded off to the nearest 0.5 cm), weight (rounded off to the nearest 0.1 kg) and mean systolic and diastolic pressure (average of three readings). Body mass index was calculated as weight (kilograms) divided by squared height (meters^2). Fasting blood samples were collected and immediately transferred to a nonheparinized tube for centrifugation. Collected serum was then transferred to pre-labeled tubes, stored in liquid nitrogen and delivered at the University of Southampton, UK. The samples were subjected to analysis upon arrival without further storage.

Biochemical analyses

Fasting glucose and lipid profile were measured using a chemical analyser (Konelab, Espoo, Finland). Serum 25(OH)D levels were measured by a specific enzyme-linked immunosorbent assay (IDS, Tyne and Wear, UK). The inter- and intra-assay variability of this assay was 5.2% and 4.5%, respectively.

Clinical data analysis

Clinical data were analysed using the Social Sciences version 16.0 (SPSS, Chicago, IL). All parameters are presented as mean ± standard deviation. A paired Student T-test was used to compare within group differences between baseline and 12 months after the intervention. Significance was set at p-value ≤ 0.05.

Serum procurement and proteomic analysis

The procurement and handling of sera was in accordance to the recommendations of the Standard Operating Procedure Integration Working Group (SOPIWG) [25]. The serum specimens were thawed and vortexed for 2 minutes. At baseline and after the 12-month intervention, individual 50 µL aliquots from all female participants were randomly selected and combined in two master pools as biological replicates (n=12 per pool at baseline and at 12-months). The same procedure was applied to sera from male participants (n=13 per pool at baseline and at 12-months). From each of the eight groups (Females Baseline 1, Females Baseline 2, Females End-of-Study 1, End-of-Study 2, Males Baseline 1, Males Baseline 2, Males End-of-Study 1, Males End-of-Study 2) unprocessed serum was subjected to depletion-free, quantitative serum proteomic analysis using our previously published methodology [10, 18, 19]. Briefly, a 100 µL aliquot from each master pool was mixed with 400 µL mobile phase solution (6 M guanidine hydrochloride in 9:1 V/V water: methanol) and subjected to high-performance Size Exclusion Chromatography. The separated protein segments were dialysis purified and lyophilized to dryness at 4 °C. An amount of 100µg of protein from each of the corresponding master pools was subjected to trypsin proteolysis and their proteolytic peptides were iTRAQ labeled, pooled, and offline fractioned with high pH C_8 reverse phase chromatography. Each fraction was then analysed with ultra-high performance low pH C_{18} nano-liquid chromatography – high-resolution tandem mass spectrometry with the FT-Orbitrap platform as reported before [26-30].

Unprocessed raw files were submitted to Proteome Discoverer 1.4 for target decoy searching against the UniProtKB Homo Sapiens database (release date 10-Jan-2015) with SequestHT as reported before. Reporter ion ratios from unique peptides only were considered for the quantitation of the respective protein. Quantification ratios were median-normalized and $\log_2$ transformed. A protein was considered differentially expressed between the male and female groups after intervention relative to baseline when its 2-group T-Test p-value was ≤ 0.05. Only proteins identified with at least two unique peptides were further considered for bioinformatics analysis in compliance with the Paris Publication Guidelines for the analysis and documentation of peptide and protein identifications

(http://www.mcponline.org/site/misc/ParisReport_Final.xhtml) and the recently published guidelines for high-confidence protein identification [31]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [32] partner repository with the dataset identifier PXD003663.

Principal component analysis and protein hierarchical clustering analysis

Principal component analysis using the reporter ion ratios of all analyzed proteins after the 12-month intervention compared to baseline was performed using BioConductor-R (version 2.15.1) and g-plots in R (version 3.1.2). Hierarchical clustering analysis visualized in heatmap format using the differentially expressed proteins between the male and female groups after the 12-month intervention compared to baseline was performed using the Cluster 3.0 (http://bonsai.hgc.jp/ mdehoon/software/cluster/software.htm) and the Java Treeview software (http://jtreeview.sourceforge.net). The clustering parameters were iteratively determined to be: correlation (Euclidean distance) metrics and complete linkage method using the normalized log2ratios for each protein.

Pathway Map analysis

The *in silico* functional interpretation of the modulated serum proteome between the male and female groups was conducted with the pathway map analysis feature of MetaCore (GeneGo, St. Joseph, MI, US). A hypergeometric test was used for the identification of canonical pathway maps that were enriched in the differentially expressed serum proteins between sexes (Benjamini−Hochberg FDR-corrected, p-value ≤ 0.05).

ELISA measurements

ELISA measurements for the proteins vitamin-K dependent protein C (MyBioSource, catalogue number MBS5703600, dilution 1:200), von Willebrand factor (Abcam, Cambridge, UK, catalogue number ab108918), fibrinogen gamma chain (Abcam, Cambridge, UK, catalogue number ab108842, dilution 1:1000) and multimerin-1 (USCN, catalogue number SEC622Hu, dilution 1:5) were performed on individual serum samples of females and males at baseline and after the 12-month intervention following the manufacturer's instructions. These subjects were randomly selected from the cohort using the randomization function of Excel, Microsoft Office 2011. Due to insufficient sample volume from certain subjects, the number of serum samples analysed for each ELISA measurement were as follows: vitamin-K dependent protein C (n=23 females and n=23 males); von Willebrand factor; Fibrinogen gamma chain (n=20 females and n=20 males) and multimerin-1 (n=22 females and n=22 males). Delta values of change (end-of-study minus baseline) were compared between female and male participants using a heteroscedastic two-group T-Test. Significance was set at p-value ≤ 0.05.

Results

The participants' clinical characteristics, sun exposure, physical activity and nutritional assessment data at baseline and after the 12-month intervention are detailed in **Table 1.** Serum 25(OH)D levels was the only assessed clinical indicator that changed significantly as a result of the intervention. With the exception of vitamin D intake and sun exposure, there was no significant change in physical activity levels, total energy, macronutrient and micronutrient intake between baseline and end-of-study for both female and male groups (**Table 1**).

Only proteins identified at ≥ 95% confidence (FDR corrected p-value ≤ 0.05 at the peptide level) were accounted for. A total of 1,841 unique proteins were fully quantified across all eight conditions of the multiplex experiment (**Supplementary Table 1**). Principal component analysis of

the reporter ion ratios of analysed proteins showed that females clustered separately from males (**Figure 1A**), suggesting that vitamin D status improvement exerts sexually dimorphic effects at the serum protein level.

A total of 282 proteins identified with at least two unique peptides were differentially expressed after the intervention between females and males (p-value ≤ 0.05) (**Supplementary Table 2**). These are visualized in heatmap format in **Figure 1B**. The blood coagulation pathway was significantly enriched in the differentially expressed proteins between the two sexes after the vitamin D intervention (FDR corrected p-value = 9.9E-22) (**Figure 2**). Representative proteins of the blood coagulation pathway were measured with ELISA to individual serum specimens within the same clinical cohort (delta value = end-of-study minus baseline): vitamin-K dependent protein C [Mean delta value ± SD: -3.2±11.0 ng/mL in females and 2.2±6.5 ng/mL in males; p-value = 0.05 between sexes], von Willebrand factor [Mean delta value ± SD: 120.8±288.2 U/L in females and -62.5±294.1 U/L in males; p-value =0.04 between sexes], fibrinogen gamma chain [Mean delta value ± SD: 2.6±6.9 µg/mL in females and -0.6±1.4 µg/mL in males; p-value =0.05 between sexes] and multimerin-1 [Mean delta value ± SD: -1.2±5.6 ng/mL in females and 12.8±29.6 ng/mL in males; p-value =0.04 between sexes] (**Figures 2 and 3**) (**Supplementary Table 3**). Their ELISA measurements were in agreement with the differential expression observed with serum proteomics: vitamin-K dependent protein C [Mean iTRAQ log2ratio end-of-study/baseline ± SD: 0.09±0.09 in females and 0.41±0.16 in males; p-value =0.02 between sexes], von Willebrand factor [Mean iTRAQ log2ratio end-of-study/baseline ± SD: -0.05±0.06 in females and -0.21±0.07 in males; p-value =0.01 between sexes], fibrinogen gamma chain [Mean iTRAQ log2ratio end-of-study/baseline ± SD: 0.03±0.13 in females and -0.37±0.06 in males; p-value =0.001 between sexes] and multimerin-1 [Mean iTRAQ log2ratio end-of-study/baseline ± SD: -0.16±0.24 in females and 0.29±0.08 in males; p-value =0.03 between sexes].

Discussion

The present non-targeted serum proteomics prospective interventional study showed that vitamin D status improvement among overweight adults exerts sex-specific effects on proteins participating in the blood coagulation pathway. To date, only few studies have examined the sex specificity of the pleiotropic actions of vitamin D. A recent cross-sectional study reported sexually dimorphic trends between vitamin D status and its role in conditioning the severity of coronary artery disease [33]. Interestingly, this study observed that lower 25(OH)D levels played a more pronounced role in conditioning the severity of coronary artery disease in females compared to males. Another cross-sectional study examined the association between vitamin D deficiency and the associated risk of developing obesity-related complications, including type II diabetes mellitus (T2DM) and insulin resistance [9]. After adjusting for confounding factors, including BMI, the study showed an inverse association of 25(OH)D with T2DM only in females, suggesting a sex-specific extra-skeletal effect of vitamin D. We have previously reported that vitamin D supplementation in patients with T2DM improved serum concentrations of magnesium and selenium in a sex-specific manner [34].

Two different parameters could in isolation or synergistically explain this sexually dimorphic vitamin D action and its effect to blood coagulation. Firstly, our group has shown previously that among overweight/obese adults, males exhibited higher circulating levels of proteins involved in the blood coagulation pathway relative to females [10]. Secondly, this same study provided protein level evidence of the increased conversion of 25-hydroxyvitamin D to the active metabolite 1,25-hydroxyvitamin D in men compared to women. These traits may be linked to the effects of sex hormone metabolism on vitamin D disposition [35, 36] and the blood coagulation system [37-39]. In accordance to the current mandates of the U. S. National Institutes of Health (NIH), sex specific differences in the metabolism and disposition of vitamin D should be taken into consideration in cell and animal studies [40].

A potential limitation to the experimental study design was the sample pooling for each clinical condition examined. To overcome this limitation, key proteins were validated with ELISA in individual serum samples from the same clinical cohort. Another limitation to this study is the physiological interpretation of the sexual dimorphic trend observed. Such an investigation was beyond the scope of this study. Finally, the number of subjects that participated in the present proof-of-principle study is rather small and a non-treated control group was not included, thus further confirmation of our findings in randomized control trials including larger number of normal weight as well as overweight/obese subjects is warranted.

Conclusions and Future Outlook

The quantitative serum proteomics approach used in this study revealed a sexually dimorphic effect of vitamin D status correction on proteins participating in blood coagulation among overweight adults. These sex-mediated vitamin D effects should be factored in the design and interpretation of vitamin D observational and interventional studies testing cardiometabolic outcomes.

Acknowledgments

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Table 1. Clinical characteristics of study subjects

	Females		Males	
	Baseline	**Follow-Up**	**Baseline**	**Follow-Up**
N	24		26	
Age (years)	26.2 ± 4.4		30.3 ± 4.6	
BMI (kg/m^2)	27.6 ± 1.9	27.2 ± 2.8	28.4 ± 1.6	27.5 ± 2.2
Sun exposure (hr/week)	1.1 ± 0.2	4.2 ± 0.3*	0.5 ± 0.3	3.5 ± 0.4**
Physical activity (MET-min/week)	540 ± 280	490 ± 315	702 ± 308	650 ± 240
Energy intake (kcal/d)	1841 ± 880	1894 ± 520	2059 ± 210	2082 ± 195
Carbohydrate intake (g/d)	294 ± 138	310 ± 140	333 ± 130	339 ± 125
Fibre intake (g/d)	17 ± 10	18 ± 9	16.5 ± 8.5	17 ± 10
Protein intake (g/d)	73 ± 31	69 ± 28	75 ± 35	77 ± 32
Fat intake (g/d)	49 ± 23	50 ± 27	55 ± 40	54 ± 38
Dietary SFA intake (g/d)	15.7 ± 9.0	16.2 ± 8.8	18.7 ± 11.4	18.0 ± 12.6
Dietary MUFA intake (g/d)	17.3 ± 9.4	16.9 ± 8.5	19.1 ± 12.7	19.5 ± 11.6
Dietary PUFA intake (g/d)	11.5 ± 7.0	12.0 ± 6.5	13.4 ± 10.1	13.0 ± 10.5
Dietary calcium intake (mg/d)	910 ± 350	872 ± 295	785 ± 180	805 ± 223
Dietary vitamin D intake (μg/d)	0.6 ± 0.2	21.1 ± 4.7**	1.2 ± 0.5	19.3 ± 6.4**
Systolic blood pressure (mmHg)	115.0 ± 13.8	118.0 ± 9.5	122.0 ± 14.8	110.0 ± 10.0
Diastolic blood pressure (mmHg)	80.0 ± 10.9	80.0 ± 4.3	77.0 ± 6.7	73.0 ± 5.7
Glucose (mmol/L)	5.4 ± 1.23	5.6 ± 1.07	5.8 ± 0.75	5.9 ± 0.74
Triglycerides (mmol/L)	1.69 ± 0.70	2.2 ± 1.1	1.8 ± 0.66	1.4 ± 0.46

Total-Cholesterol (mmol/L)	5.4 ± 1.23	5.6 ± 1.07	5.3 ± 0.55	4.8 ± 0.43
HDL-Cholesterol (mmol/L)	0.96 ± 0.25	0.92 ± 0.24	0.75 ± 0.24	0.94 ± 0.21
LDL-Cholesterol (mmol/l)	0.43 ± 0.24	0.34 ± 0.28	0.48 ± 0.11	0.48 ± 0.08
25(OH)D (nmol/L)	17.4 ± 6.7	65.4 ± 14.4**	19.2 ± 5.2	61.6 ± 9.8**

Note: Data presented as mean ± standard deviation; * denotes significance at 0.05 level; ** denotes significance at 0.01 level; p-value significant at < 0.05. A paired T-test was performed to compare differences between baseline and after 1 year intervention. Abbreviations: MET (metabolic equivalent of task); SFA (saturated fatty acids); MUFA (mono-unsaturated fatty acids); PUFA (poly-unsaturated fatty acids); HDL (high-density lipoprotein); LDL (low-density lipoprotein).

Figure Legends

Figure 1. A. Principal component analysis of the reporter ion ratios of all profiled proteins showed that females clustered separately from males following the 12-month vitamin D intervention. **B.** Hierarchical clustering analysis visualized in heatmap format of differentially expressed serum proteins between females and males following the 12-month vitamin D intervention (p-value ≤ 0.05)

Figure 2. The blood coagulation pathway was found to be significantly enriched in the differentially expressed proteins between males and females following the 12-month vitamin D intervention. Analysed proteins are denoted with a thermometer. Red=up-regulation; Blue=down-regulation; F=Females end-of-study vs. baseline; M=Males end-of-study vs. baseline. Circled proteins were verified with ELISA in individual samples.

Figure 3. Proteomic results (iTRAQ log2ratios of unique peptides: end-of-study vs. baseline) for the biological replicates of pooled samples (n=24 females; n=26 males) and respective validation with ELISA (delta value: end-of-study minus baseline) in individual serum samples of females and males (n=20 to 23) for: A. Vitamin-K dependent protein C [Proteomics: mean iTRAQ log2ratio ± SD: 0.09±0.09 in females and 0.41±0.16 in males; p-value =0.02 between sexes; ELISA: mean delta value ± SD: -3.2±11.0 ng/mL in females and 2.2±6.5 ng/mL in males; p-value =0.05 between sexes], von Willebrand factor [Proteomics: mean iTRAQ log2ratio ± SD: -0.05±0.06 in females and -0.21±0.07 in males; p-value =0.01 between sexes; ELISA: mean delta value ± SD: 120.8±288.2 U/L in females and -62.5±294.1 U/L in males; p-value =0.04 between sexes], fibrinogen gamma chain [Proteomics: mean iTRAQ log2ratio ± SD: 0.03±0.13 in females and -0.37±0.06 in males; p-value =0.001 between sexes; ELISA: mean delta value ± SD: 2.6±6.9 μg/mL in females and -0.6±1.4 μg/mL in males; p-value =0.05 between sexes] and multimerin-1 [Proteomics: mean iTRAQ log2ratio ± SD: -0.16±0.24 in females and 0.29±0.08 in males; p-value =0.03 between sexes; ELISA: mean delta value ± SD: -1.2±5.6 ng/mL in females and 12.8±29.6 ng/mL in males; p-value =0.04 between sexes].

Figure 1

Principal Component and Hierarchical Clustering Analysis

A

● =Females end-of-study vs. baseline (biological replicates)

● =Males end-of-study vs. baseline (biological replicates)

B

Females end of study vs. baseline (biological replicates)

Males end of study vs. baseline (biological replicates)

Blood coagulation pathway (*p-value* = 1.84E-15)

Up-regulated after vitD intervention vs. baseline

Down-regulated after vitD intervention vs. baseline

F= Females
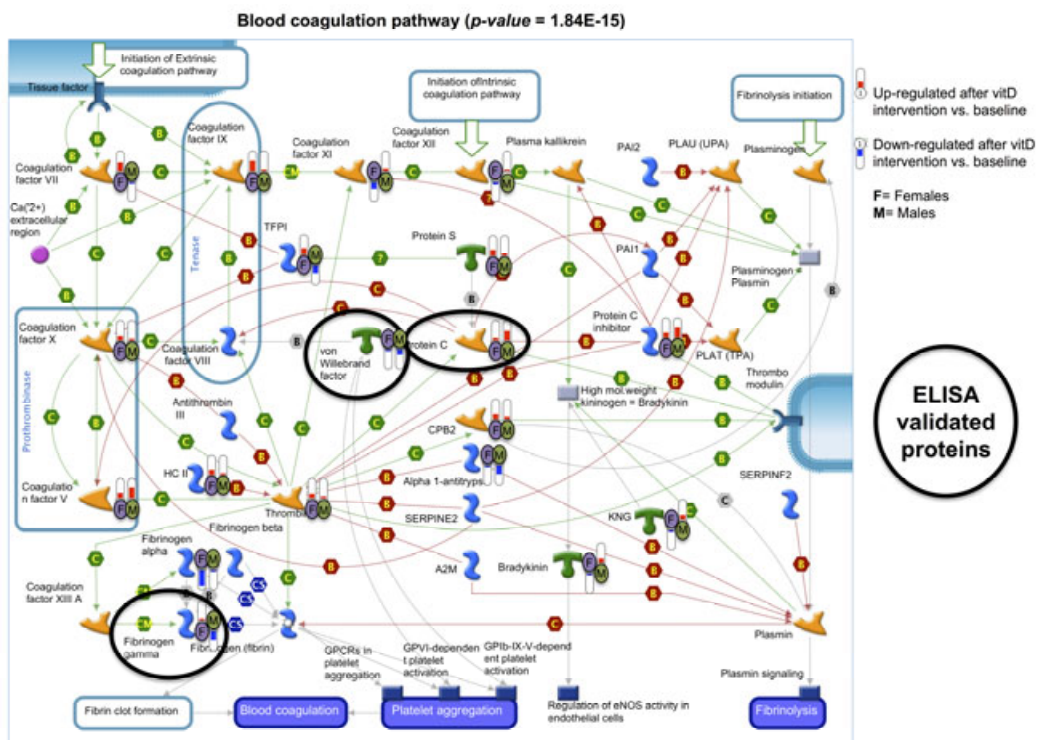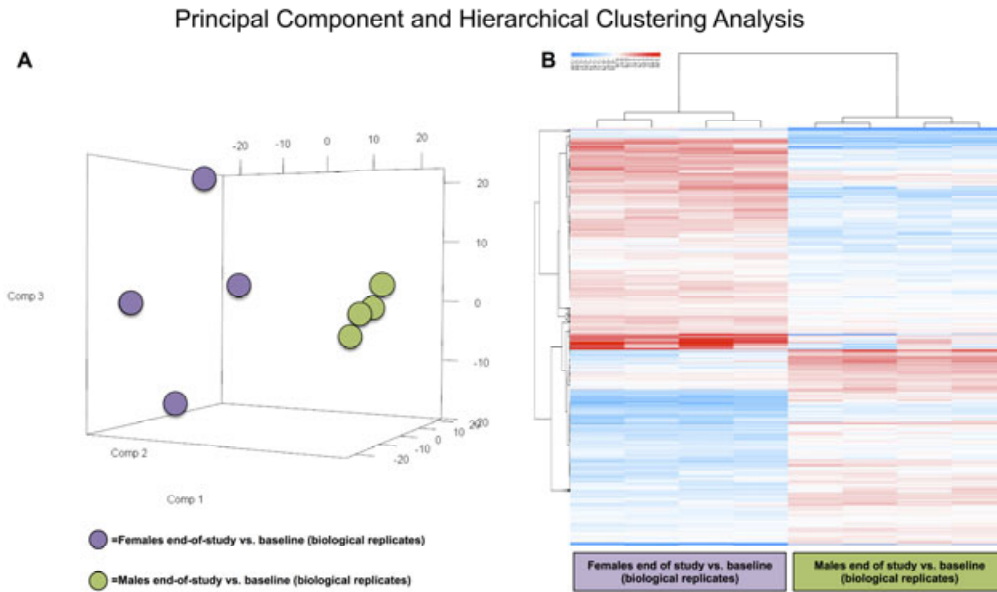M= Males

ELISA validated proteins

Figure 2

Validation of key proteins participating in blood coagulation with ELISA