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A novel, multiplexed targeted mass spectrometry assay for quantification of complement factor H (CFH) variants and CFH-related proteins 1–5 in human plasma

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Abstract

Age-related macular degeneration (AMD) is a leading cause of visual loss among older adults. Two variants in the complement factor H (CFH) gene, Y402H and I62V, are strongly associated with risk of AMD. CFH is encoded in Regulator of Complement Activation gene cluster in chromosome 1q32, which includes complement factor-related (CFHR) proteins, CFHR1 to CFHR5, with high amino acid sequence homology to CFH. Our goal was to build a selected reaction monitoring (SRM) assay to measure plasma concentrations of CFH variants Y402, H402, I62, and V62, and CFHR1–5. The final assay consisted of 24 peptides and 72 interference-free SRM transition ion pairs. Most peptides showed good linearity over 0.3–200 fmol/ μ L concentration range. Plasma concentrations of CFH variants and CFHR1–5 were measured using the SRM assay in 344 adults. Plasma CFH concentrations (mean, SE in μ g/mL) by inferred genotype were: YY402, II62 (170.1, 31.4), YY402, VV62 (188.8, 38.5), HH402, VV62 (144.0, 37.0), HY402, VV62 (164.2, 42.3), YY402, IV62 (194.8, 36.8), HY402, IV62 (181.3, 44.7). Mean (SE) plasma concentrations of CFHR1–5 were 1.63 (0.04), 3.64 (1.20), 0.020 (0.001), 2.42 (0.18), and 5.49 (1.55) μ g/mL, respectively. This SRM assay should facilitate the study of the role of systemic complement and risk of AMD.

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Keywords

age-related macular degeneration; complement factor H; complement factor H-related proteins; mass spectrometry; selected reaction monitoring

1 Introduction

Age-related macular degeneration (AMD) is the leading cause of visual loss among adults aged 65 years or older in developed countries [1]. Genetic factors, smoking, and diet account for most of the risk of AMD [1]. The complement factor H gene, *CFH*, is strongly associated with AMD [2–5]. The complement system plays an important role in cellular integrity, microbial killing, immune surveillance, tissue homeostasis, and mediation of inflammatory responses [6]. Complement is involved in the recognition of diseased or damaged host cells, regulation of cellular immune responses, and interaction with the coagulation cascade. Complement discriminates between foreign, altered, and healthy surfaces. Excessive activation or insufficient control of the complement system appears to increase the risk of AMD, but the biological mechanisms remain unknown.

There are four common variants in *CFH*, of which two are strongly associated with risk of AMD: tyrosine 402 to histidine (Y402H) and isoleucine 62 to valine (I62V). *CFH* is encoded in the Regulator of Complement Activation gene cluster in chromosome 1q32, which also includes five complement factor-related (*CFHR*) proteins, *CFHR1* to *CFHR5*, with high amino acid sequence homology to *CFH*. A common haplotype, with deletion of *CFHR1* and *CFHR3* (CNP147 deletion), is associated with a decreased risk of AMD [7,8]. The protective effects associated with the CNP147 deletion are independent of the *CFH* Y402H polymorphism [9]. Variants in *CFHR2*, *CFHR4*, and *CFHR5* have also been associated with risk of AMD [10,11]. The substitution of histidine for tyrosine at residue 402 alters the binding of *CFH* and impairs the function of *CFH* in limiting inflammation [12–17]. *CFH* binding and function are also compromised by the substitution of valine for isoleucine at residue 62 [18–20].

AMD is characterized by localized inflammation and the presence of multiple proteins associated with complement, complement activation and regulation, and inflammatory proteins in drusen and the retinal pigment epithelium, Bruch's membrane, and choriocapillaris [21,22]. The relative roles of systemic versus local production of *CFH* in the pathogenesis of AMD remain unclear. Whether plasma concentrations of *CFH* Y402H and I62V variants and plasma *CFHR1–5* concentrations are related to the risk of AMD has not been established. Currently available antibody and aptamer-based approaches are limited in distinguishing and quantifying highly homologous sequence variants of *CFH* and *CFHR1–5*. SRM, however, overcomes the shortcomings of current immunoassays by providing a highly sensitive and specific targeted mass spectrometric methodology for precise and accurate quantification of proteins and variants. Because of the high sequence homology among *CFH* variants and *CFHR1–5*, the optimal approach for characterization of these proteoforms in plasma is through use of SRM assay (Supporting Information Fig. 1). In this paper, we describe a novel SRM assay that can accurately measure plasma *CFH* variants and *CFHR*

proteins. The assays were processed through the PeptideAtlas SRM Experiment Library (PASSEL) and made available at <http://www.peptideatlas.org/PASS/PASS00914>.

2 Materials and methods

2.1 Selection of proteotypic peptides

For SRM assay development, tryptic peptides were selected following the guidelines of Kuzyk and colleagues [23]. Tryptic peptides unique to each protein were identified using PeptideCutter (ExPASy, Swiss Institute of Bioinformatics), NCBI BLAST and UniProt/BLAST searches, with further support for selection of peptides and optimization of transitions through Skyline (Seattle Proteome Center) using the ProteoWizard libraries (Table 1).

2.2 Synthesis and purification of peptides

Peptides were obtained from New England Peptide (Gardner, MA). Tryptic fragment peptides were prepared by Fmoc-based solid-phase peptide synthesis using per-15N, 13C-labeled (>99% isotopic purity) Arg or Lys as the C-terminal residue attached to the resin. Cysteine side-chain residues were blocked as the carboxyacetylthiomethyl thioether. Peptides were cleaved from the resin with ~90% trifluoroacetic acid (TFA) containing appropriate scavengers and isolated by precipitation from ether or by drying of the cleavage cocktail. Peptides were purified by reversed phase chromatography (C18 stationary phase using water-acetonitrile gradients, ion-pairing agent ~0.1% TFA). Peptide purity was confirmed by analytical HPLC. MALDI-MS was used to confirm peptide identity. Purified peptide solutions were prepared and the concentration of the solution was determined by amino acid analysis.

2.3 Optimization of the assay

Selection of optimal charge state and collision energy, confirmation of co-elution of endogenous and SIS peptides, and interference detection was conducted as detailed elsewhere [23]. Three interference-free SRM ion pairs constituted the final SRM assay for the respective proteotypic peptides. The SIS peptide spiking concentration was optimized, and calibration curves, linear range of quantification, and lower limit of quantification are shown in Supporting Information Tables 1 and 2.

2.4 Study samples

Plasma samples were obtained from 344 adult who were participants in the Age, Gene/Environment Susceptibility-Reykjavik (AGES-Reykjavik) Study, a population-based study aimed at identifying factors that contribute to disease in older adults. The design and assessment of the cohort are described in detail elsewhere [24]. In 2002, when the AGES-Reykjavik Study began, 11,549 previously-examined members of the Reykjavik study cohort (1967–1996) were still alive. A random sample of 5,764 individuals was examined for the AGES-Reykjavik Study in 2002–2006 (known as the AGES I study visit) [24]. This protocol was approved by the Johns Hopkins School of Medicine Institutional Review Board.

2.5 SRM assay development

SRM assays were run on a 5500 triple quadrupole (QTrap) mass spectrometer (Sciex, Framingham, MA) with an instrument run time of 20 min/sample including the 5 min column regeneration step. Samples were run in a randomized manner in blocks of 30 with strict attention to QC. Each block of 30 contained 27 study samples and 3 internal plasma standards in order to monitor QC of each block run. At the beginning and end of each large sample run consisting of ten blocks of 30 samples, a series of SIS peptide standards (13 point standard curve) are run first, followed by a block of 30 samples. Between each block of 30 samples, a mini series of SIS peptide standards (3 QC samples) was run. After completion of ten blocks, the series of SIS peptide standards (13 points) was repeated. Standards and samples were run with three technical replicates for calculation of mean and CVs. Any sample block with CV >20% was re-run with the same standard curves and QC arrangement. The target variants and protein concentrations were calculated and normalized by the linear equation of their respective transitions in a plasma matrix, which was aliquoted from 200 adult participants.

2.6 Statistical analysis

We estimated the mean concentrations of CFHR1–5 and of CFH stratified by genotype. Presence of an allele (I or V at residue 62; Y or H at residue 402) was defined for each participant as detection of at least one variant-specific transition (precursor/fragment ion pair) in two or more technical replicates. For example, if the same I-specific transition was detected in at least two technical replicates, but no V-specific transition was detected in at least two technical replicates, then the participant was inferred to have genotype II62. We used analogous logic to infer genotype VV62. If both an I-specific and V-specific transition were detected in at least two technical replicates, then the participant was inferred to have genotype IV62. We used analogous logic to infer YY402, HH402, and YH402 genotypes. Separately by genotype, we used a linear mixed-effects model framework to estimate mean CFH concentrations. For double homozygotes (e.g., VV62, HH402), we fit an intercept-only model with two random effects: one for participant nested within plate, within transition, and within peptide; and another for participant nested within plate. The first random effect addressed peptide- and transition-specific heterogeneity between plates and participants whereas the second random effect addressed overall heterogeneity between plates and participants. For heterozygotes, we fit a mixed-effects model with intercept and variant-specific indicator fixed effects. Next, we estimated the sum of mean variant-specific peptides at the heterozygous location and used an inverse-variance weighted analysis to compute the overall CFH mean and a first-order Taylor-series expansion to compute the standard error. For example, the model for IV62, YY402 participants included fixed effects for the intercept, I-specific transitions, and V-specific transitions. The mean peptide at residue 62 was computed as the sum of mean I-specific peptide and V-specific peptide. Mean CFH was computed as the mean of residue 62 peptide and all other CFH peptides (the intercept) using inverse-variance weighting. For CFHR1–5, we fit an intercept-only mixed-effects model for each protein with a random effect for participant nested in plate. All analyses were performed using R software version 3.2.0 [27]. Mixed effects models were fit using the lmer() function from the lme4 package [28].

3 Results

The first step in assay development was to identify the best responding peptides of unique sequence (the signature peptides). For quantifying proteins, we had designed and synthesized 40 peptides involved in three CFH, four SNP variants of V62, I62, H402 and Y402, and five CFHR1–5 proteins, including natural and stable-isotope-labeled peptides.

We optimized and validated all peptides in pure buffer of 0.1% formic acid and plasma matrix by using 5500 QTrap platform prior to the SRM assay, and no significant matrix effect was found for these peptides. Figure 1 shows the representative MS/MS spectra of CFH proteins with light and heavy peptides respectively: a) and d) CFH1_LSYTCEGGFR; b) and e) CFH2_CFEFGIDGPAIAK; c) and f) CFH3_WSSPPQCEGLPCK. A full map of spectra is shown in Supporting Information Fig. 2. Mass spectrometry optimization was conducted with a continuous injection of individual peptide and internal standard at each of 100 fmol/ μ L by ramping the parameters DP (0–400 volts), CE (5–130 volts) and CXP (0–66 volts) from low to high with a step of 1 for all parameters and a fixed setting of 10 volts for entrance potential (EP). Supporting Information Fig. 3 shows representative optimization results of collision energy for quantifying CFH variants and CFHR proteins including (A) CFHR1_ISSVGGEATF(Cam)DFPK, (B) CFHR1_ISSVGGEATF(Cam)DFPK[^] (C) CFHR2_STISAEK[^] (D) CFHR3_LGYNANTSILSFQAV(Cam)R[^] (E) CFHR4_YVT(Cam)SNGDWSEPPR[^] (F) CFHR5_GWSTPPI(Cam)SFTK[^] peptides.

The targeted peptides were separated well by an optimized HPLC method with a high resolution in plasma. Figure 2 shows a representative total ion chromatographic separation of three CFH peptides, four SNP variants, and five CFHR1–5 proteins. For each peptide, the HPLC and MS parameters of SRM assay were optimized and transitions were selected to achieve the greatest sensitivity. HPLC optimization results showed a good gradient separation of peptides can be conducted in total time of 18 min with a linear increasing concentration of acetonitrile from 5% B to 36% B within 12 min. Figure 3 shows the co-elution of heavy and natural peptides extracted-ion chromatogram (XIC). Three transitions of each peptide were found eluted exactly at the same time with the standard stable-isotope-labeled peptides respectively. We set up 36 peptides and 108 interference-free SRM transition ion pairs as the final SRM assay. Table 1 shows selected proteotypic peptide length, molecular weight, and charge status in SRM assay. Supporting Table 1 shows the full parameters to monitor peptides, including parent ion Q1 and fragment Q3, dwell time, declustering potential (DP), and peptide-specific tuned collision energy (CE), entrance potential (EP), and collision cell exit (CXP) voltages for each transition, and exact retention time (min).

Finally, linearity was evaluated by titration curves of internal standards spikes in plasma. We generated calibration curves for each SRM transition, with 13-point of concentrations at 0.00, 0.24, 0.48, 0.98, 1.95, 3.91, 7.81, 15.62, 31.25, 62.50, 125.00, 250.00, and 500.00 fmol/ μ L running in triplicate. Most peptides showed good linearity over the 0.3–200 fmol/ μ L concentration range. Supporting Information Fig. 4 shows calibration curves of the target peptides at least three transitions, all with good linear range of $R^2 > 0.99$, and intra- or inter-coefficient of variation (CV) $< 10\%$.

Using a high-resolution SRM/MRM mass spectrometry method, we determined the lowest limit of detection (LLOD) and quantification (LLOQ) for each peptide. LLOQ was defined as the lowest detected concentration with CV \leq 10% and signal-to-noise (S/N) ratio \geq 10; the instrument LLOD was based on S/N $>$ 3 (Supporting Table 2).

Detection of another variant Y402 (fragment ion y7, LLOD=9.470) was found 8 times higher than the variant H402 (double charge ion of y13, LLOD=1.184). As the highest LLOQ, we spiked the highest amount of 125 fmol/ μ L of internal standard for accurate quantification of Y402. Among five CFHR proteins, CFHR1 of the peptide ISSVGGEATF(Cam)DFPK was found on an average ion reactions with the lowest values of LLOQ=2.116 and LLOD=0.641 fmol/ μ L. Digestion efficiency and intra- and inter-plate CVs are shown in Supporting Information Fig. 5 and Supporting Information Table 3, respectively.

To measure the protein concentration in plasma, we carried out protein denaturation, reduction, alkylation, and digestion from 344 participants as a pilot testing in the AGES-Reykjavik Study. Plasma concentrations of CFH variants and CFHR1–5 were measured using the SRM assay in 344 adults (Table 2).

4 Discussion

We describe a new SRM assay for the quantification of CFH variants and CFHR1–5. To our knowledge, this study is the first SRM assay to allow measurement of these proteoform concentrations. A previous study used MALDI-TOF for detection of CFH variant but did not allow quantification [29]. The evaluation of plasma concentrations of relevant complement regulatory factors could provide insights that are not achievable from the study of genetic variants. It is reasonable to test the associations between plasma levels and the genetic variants themselves as well as determine if there is an independent association of the plasma levels of the proteins with AMD.

In this study, the mean CFH concentrations in human plasma are similar to concentration range in dried blood reported by Chambers and colleagues [30] as a SRM assay reported with IDVHLVPDR, y6⁺, 76.0 μ g/mL, SPDVINGSPISQK, b3, 81.0 μ g/mL, SSNLIILEEHLK, y6, 93.7 μ g/mL, and 116–562 μ g/mL [31] by immunoassay, but different from the plasma value of 40 μ g/mL [32] or concentration ranges 265–684 μ g/mL [33] by immunoassay or 57 μ g/mL by spectral counting [34], suggesting that a more accurate method like MS-based SRM is necessary for CFH measurements. Using variant-specific monoclonal antibodies, Hakobyan and colleagues reported mean values of CFH concentrations of 233 μ g/mL in young adults, 269 μ g/mL in elderly individuals [35], and 263 μ g/mL [36] in different control populations. Compared with CFHR1–5 concentrations with the published literature by other methods like immunoassay, our low concentration values by SRM assay are different from the estimated plasma concentration of CFHR1 (~70–100 μ g/mL), [37], CFHR2 (~50 μ g/mL) [38], CFHR3 (~70–100 μ g/mL) [39], CFHR4 (6–54 μ g/mL) [40], and CFHR5 (3–6 μ g/mL) [41]. The difference between the results of the present study using SRM and LC-MS/MS and previous studies using immunoassays might

be due to the lack of specificity of antibodies in distinguishing highly similar homologues and sequence variants such as that found in CFH and CFHR proteins.

Future work will examine the relationship of plasma CFH Y402, H402, I62, and V62 variants and CFHR1 to CFHR5 with AMD in participants in the Age, Gene/Environment Susceptibility-Reykjavik (AGES-Reykjavik) Study. This work may determine whether systemic concentrations of CFH and CFHR proteins and specific variant levels play a role in AMD and represent a potential pathway for risk stratification and/or therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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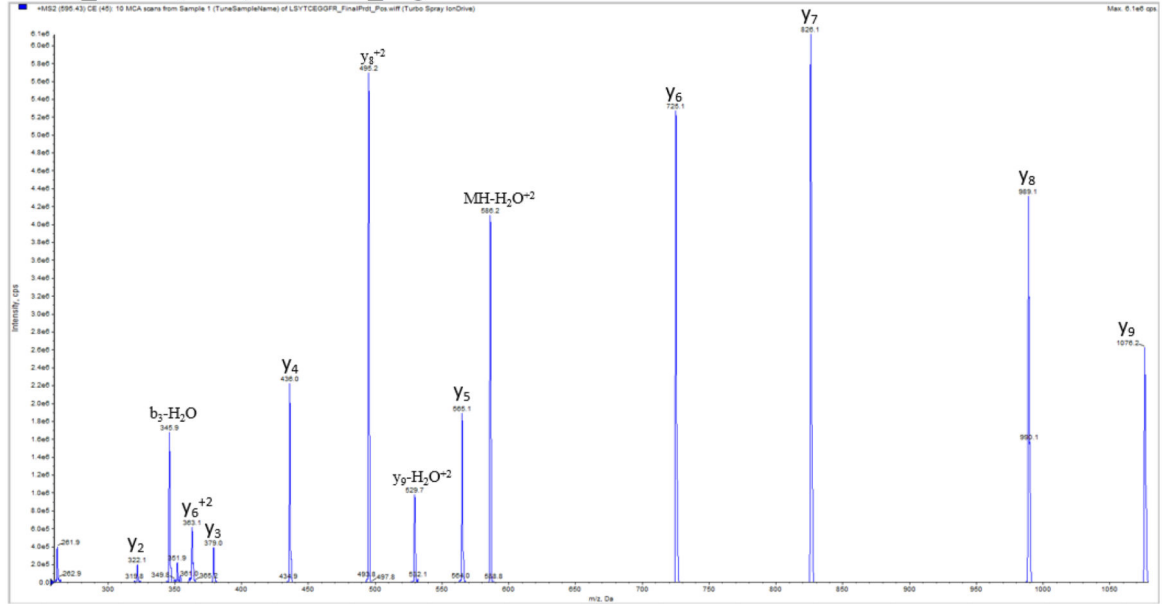
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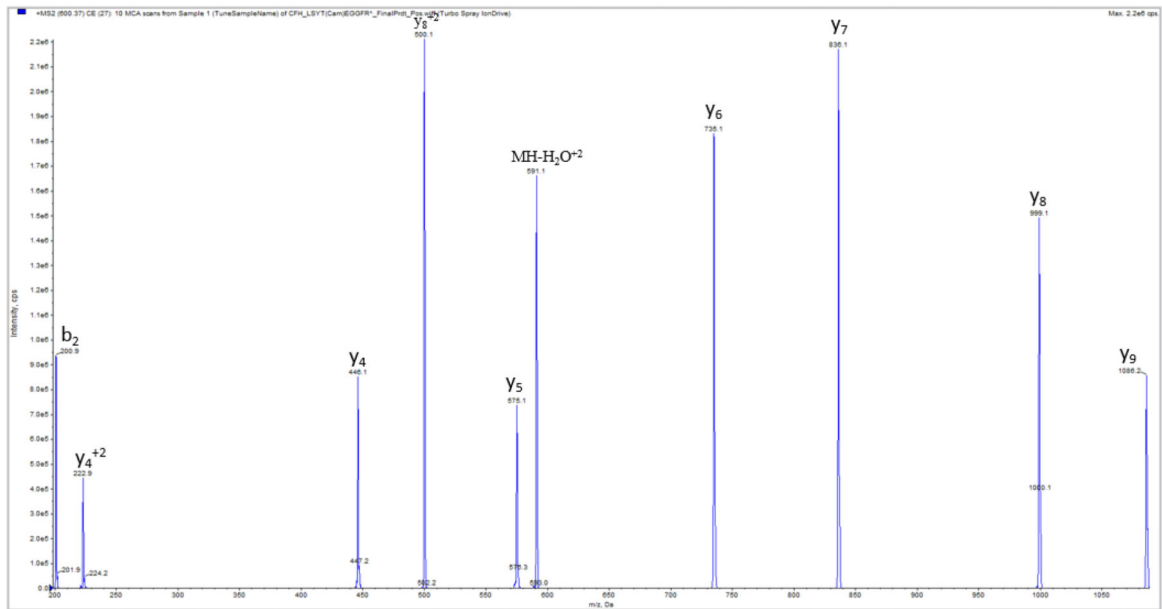
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CFH1_LSYT(Cam)EGGFR_Light MS²



a) CFH1_LSYT(Cam)EGGFR^_Heavy MS²



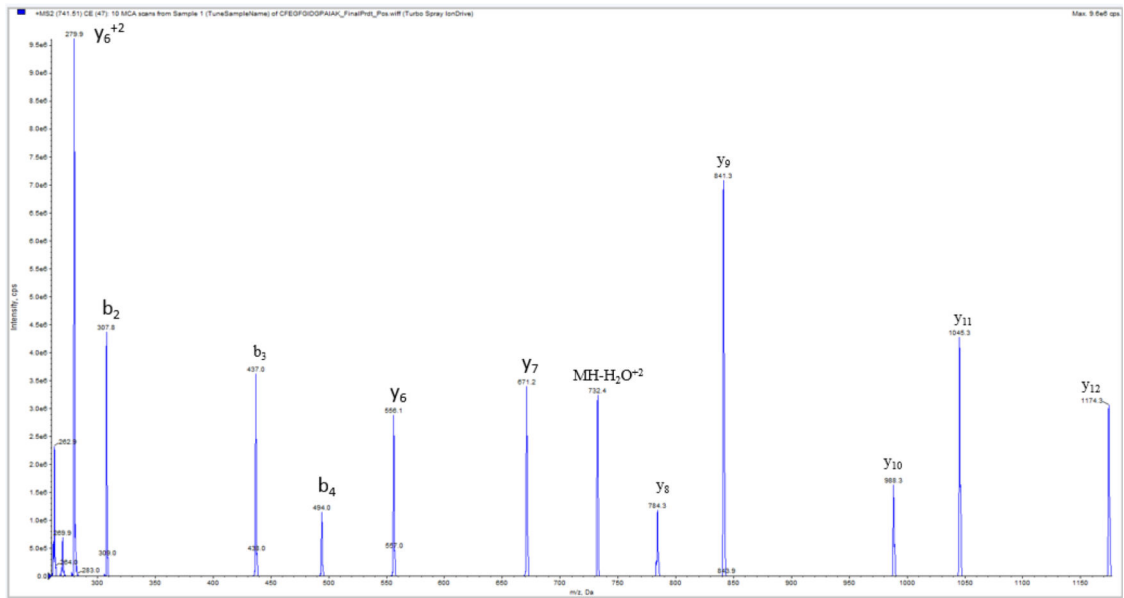
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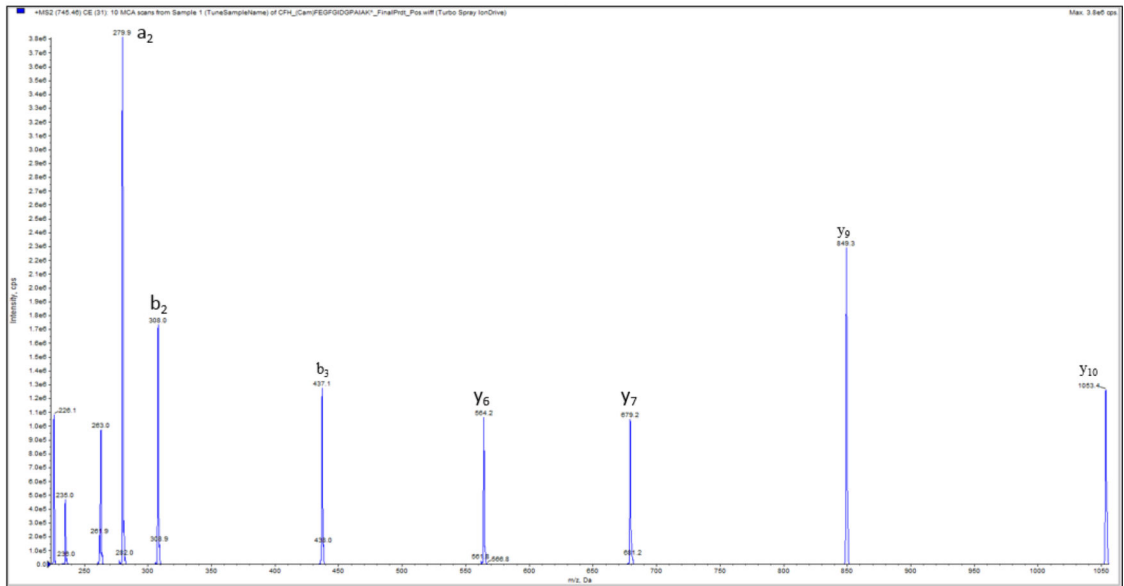
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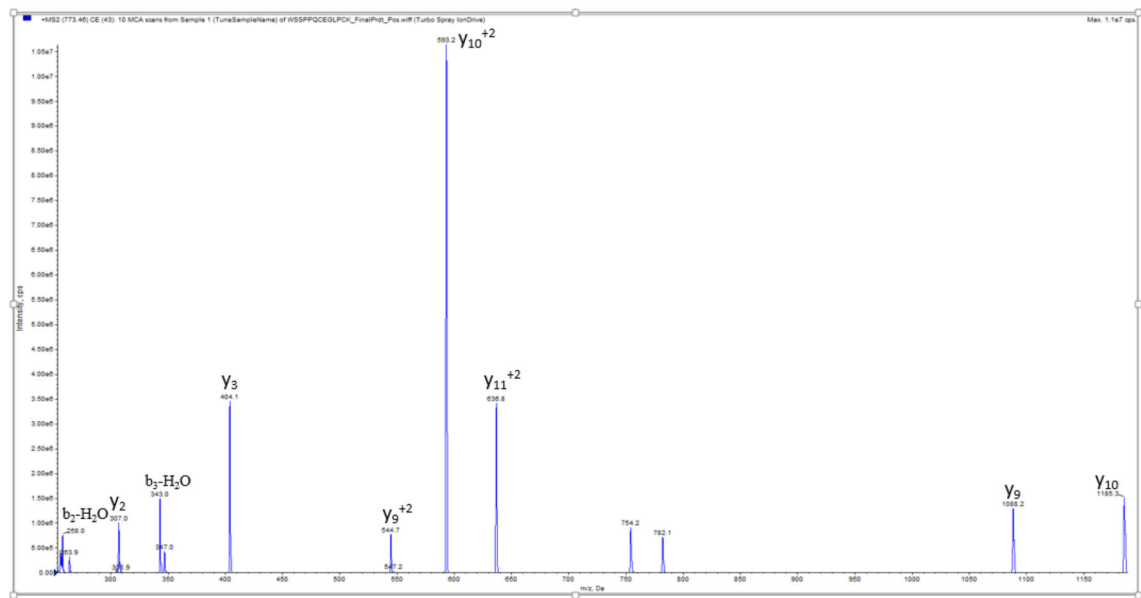
b) CFH2_(Cam)FEGFGIDGPAIAK_Light MS²



c) CFH2_(Cam)FEGFGIDGPAIAK^_Heavy MS²



d) CFH3_WSSPPQ(Cam)EGLP(Cam)K_Light MS²



e) CFH3_WSSPPQ(Cam)EGLP(Cam)K^_Heavy MS²

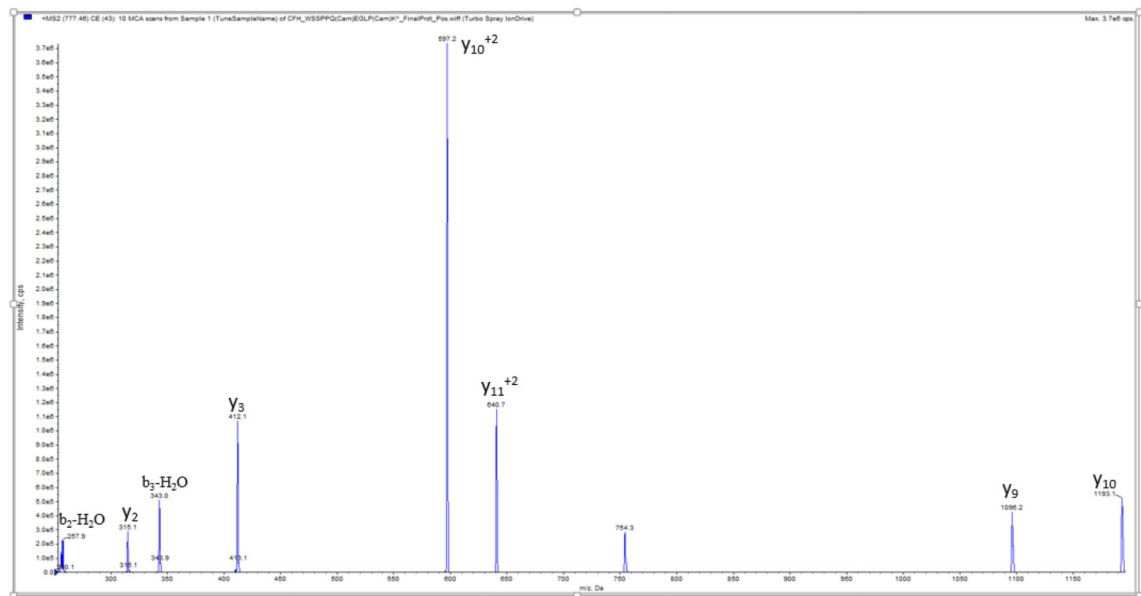


Figure 1. Representative MS/MS spectra from CFH proteins with light and heavy peptides respectively. a) and b) CFH1_LSYTCEGGFR; c) and d) CFH2_CFEFGIDGPAIAK; e) and f) CFH3_WSSPPQCEGLPCK.

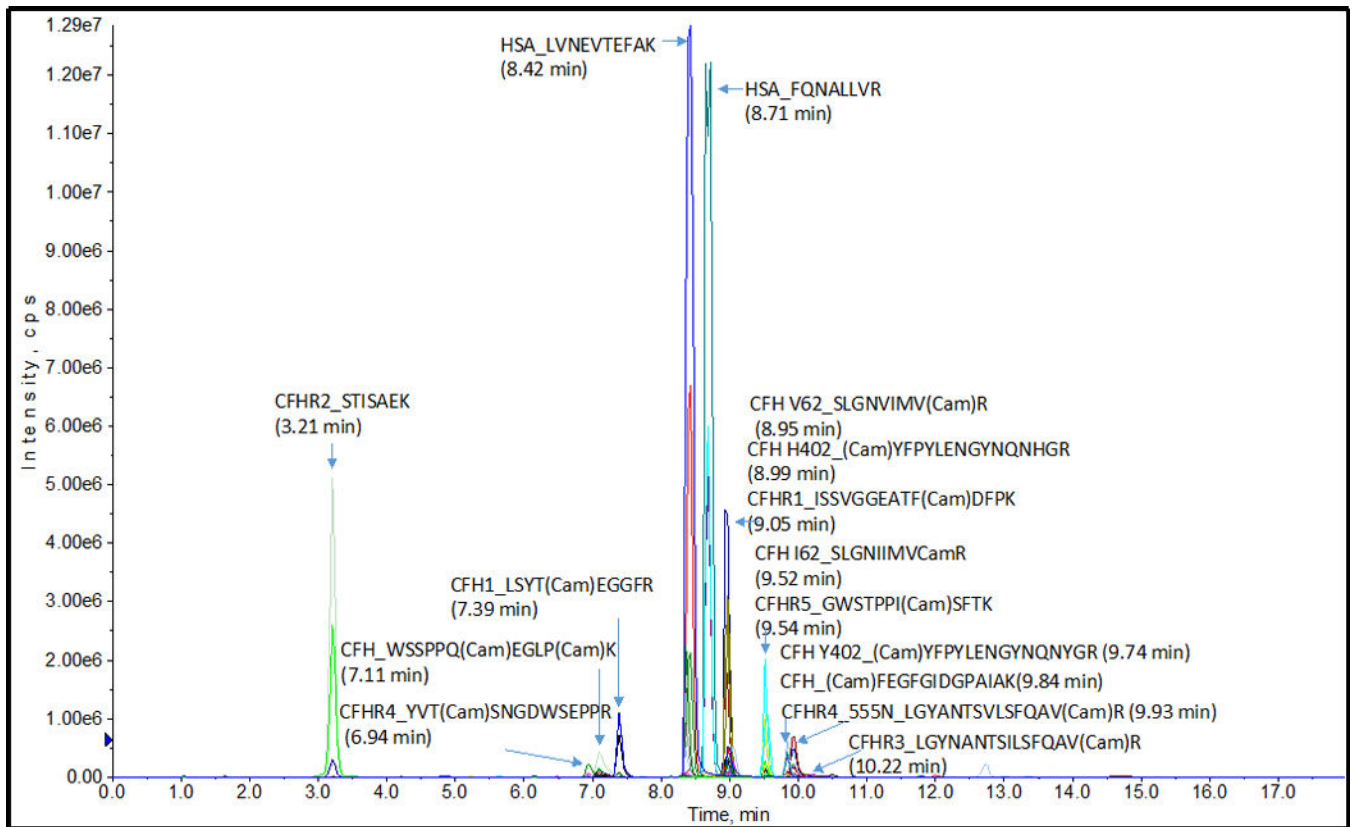
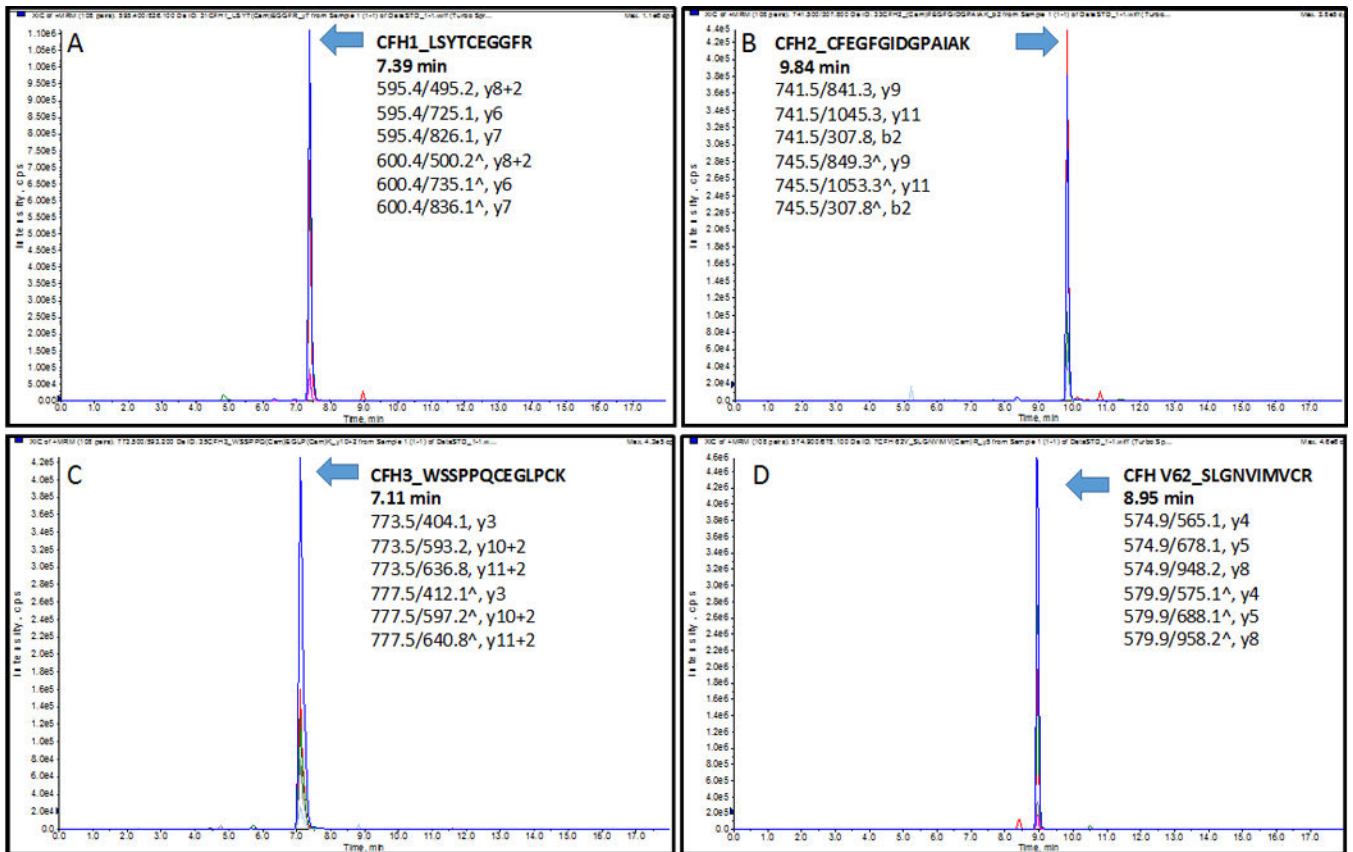
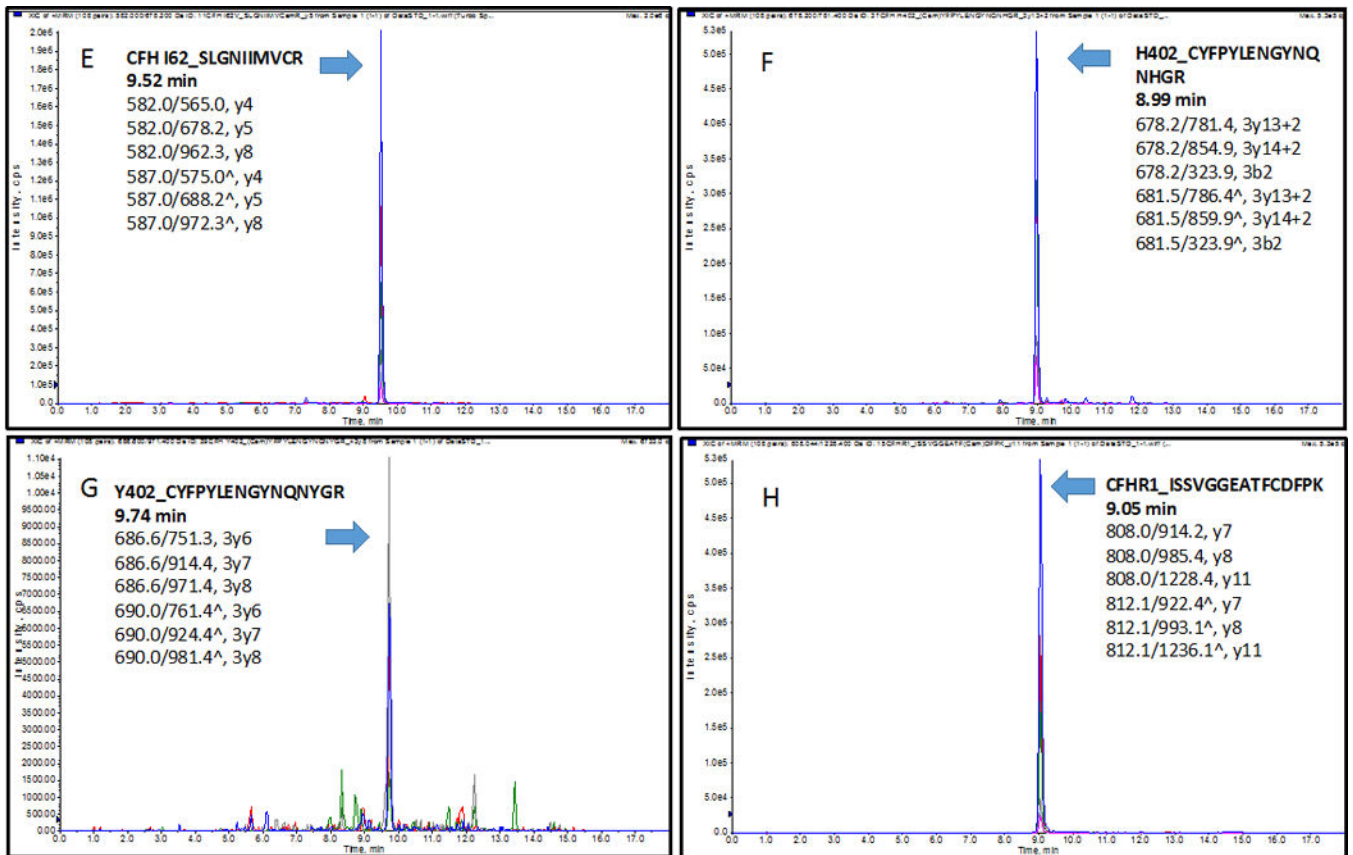


Figure 2. Representative total ion current chromatographic separation of CFH, SNP variants and CFHR1–5 proteins. HSA (human serum albumin) peptides used to monitor day-to-day variability of assay.





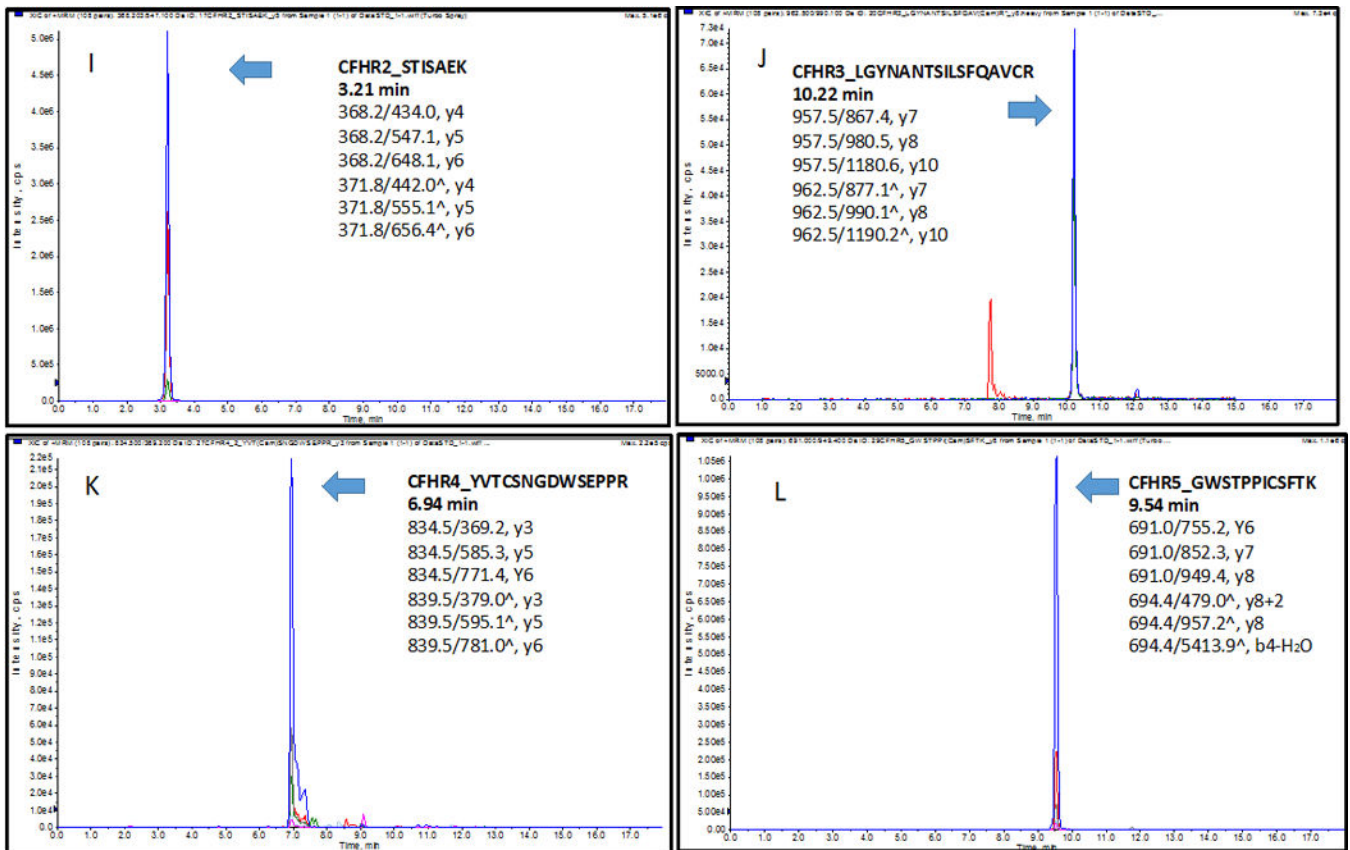


Figure 3.

The extracted-ion chromatogram (XIC) of peptides (A) CFH1_LSYTCEGGFR at 7.39 min, (B) CFH2_CFEFGFIDGPAIAK at 9.84 min, (C) CFH3_WSSPPQCEGLPCK at 7.11 min; four variants (D) V62_SLGNVIMVCR at 8.95 min, (E) I62_SLGNIIMVCR at 9.52 min, (F) H402_CYFPYLENGYNQNHGR at 8.99 min, (G) Y402_CYFPYLENGYNQNYGR at 9.74 min; (H) CFHR1_ISSVGGGEATFCDFPK at 9.05 min, (I) CFHR2_STISAEK at 3.21 min, (J) CFHR3_LGYNANTSILSFQAVCR at 10.22 min, (K) CFHR4_YVTCSNGDWSEPPR at 6.94 min, (L) CFHR5_GWSTPPICSFTK at 9.54 min. Three transitions of each peptide were found eluted exactly at the same time with the standard stable-isotope-labeled peptides respectively.

Table 1

Selection of proteotypic peptides in SRM assay

Protein name	Targeted peptide sequence in SRM	Length	Molecular weight (Da)	Charge status
CFH variant V62	SLGNVIMV(Cam)R	10	1149.8	2
	SLGNVIMV(Cam)R [^]	10	1159.8	2
CFH variant I62	SLGNIIMV(Cam)R	10	1164.0	2
	SLGNIIMV(Cam)R [^]	10	1174.0	2
CFH variant H402	(Cam)YFPYLENGYNQNHGR	16	2034.6	3
	(Cam)YFPYLENGYNQNHGR [^]	16	2044.5	3
CFH variant Y402	(Cam)YFPYLENGYNQNYGR	16	2059.8	3
	(Cam)YFPYLENGYNQNYGR [^]	16	2070.0	3
CFH	LSYT(Cam)EGGFR	10	1190.8	2
	LSYT(Cam)EGGFR [^]	10	1200.8	2
	(Cam)FEGFGIDGPAIAK	14	1483.0	2
	(Cam)FEGFGIDGPAIAK [^]	14	1491.0	2
	WSSPPQ(Cam)EGLP(Cam)K	12	1547.0	2
	WSSPPQ(Cam)EGLP(Cam)K [^]	12	1555.0	2
CFHR1	ISSVGGEATF(Cam)DFPK	14	1616.0	2
	ISSVGGEATF(Cam)DFPK [^]	14	1624.2	2
CFHR2	STISAEK	7	736.4	2
	STISAEK [^]	7	743.6	2
CFHR3	LGYNANTSILSFQAV(Cam)R	17	1915.0	2
	LGYNANTSILSFQAV(Cam)R [^]	17	1925.0	2
CFHR4	YVT(Cam)SNGDWSEPPR	14	1669.0	2
	YVT(Cam)SNGDWSEPPR [^]	14	1679.0	2
CFHR5	GWSTPPI(Cam)SFTK	12	1382.0	2
	GWSTPPI(Cam)SFTK [^]	12	1388.8	2

Note:

[^] = stable-isotope-labeled peptides; Cam=cysteine carbamidomethylation; CFH=Complement factor H; CFHR=complement factor H-related proteins

Table 2

Plasma concentrations of complement factor H variants and complement factor H-related proteins in 344 adults

Protein & inferred genotype	Sample (n)	Age ¹ (years)	Female (%)	Concentration ² (µg/mL)
CFH YY402, II62	16	79.2 (4.8)	68.8	170.1 (31.4)
CFH YY402, VV62	51	80.2 (4.5)	45.1	188.8 (38.5)
CFH YY402, IV62	56	79.0 (4.8)	60.7	194.8 (36.8)
CFH HH402, VV62	56	79.9 (5.6)	52.9	144.0 (37.0)
CFH HY402, IV62	65	79.5 (4.1)	66.1	181.3 (44.7)
CFH HY402, VV62	100	80.1 (5.9)	58.5	164.2 (42.3)
CFHR1	344	79.7 (5.1)	56.9	1.63 (0.04)
CFHR2	344	79.7 (5.1)	56.9	3.64 (1.20)
CFHR3	344	79.7 (5.1)	56.9	0.020 (0.001)
CFHR4	344	79.7 (5.1)	56.9	2.42 (0.18)
CFHR5	344	79.7 (5.1)	56.9	5.49 (1.55)

¹Mean (standard deviation)

²Mean (standard error)