Quantitative variability of 342 plasma proteins in a human twin population

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Abstract

The degree and the origins of quantitative variability of most human plasma proteins are largely unknown. Because the twin study design provides a natural opportunity to estimate the relative contribution of heritability and environment to different traits in human population, we applied here the highly accurate and reproducible SWATH mass spectrometry technique to quantify 1,904 peptides defining 342 unique plasma proteins in 232 plasma samples collected longitudinally from pairs of monzygotic and dizygotic twins at intervals of 2–7 years, and proportioned the observed total quantitative variability to its root causes, genes, and environmental and longitudinal factors. The data indicate that different proteins show vastly different patterns of abundance variation among humans and that genetic control and longitudinal variation affect protein levels and biological processes to different degrees. The data further strongly suggest that the plasma concentrations of clinical biomarkers need to be calibrated against genetic and temporal factors. Moreover, we identified 13 cis-SNPs significantly influencing the level of specific plasma proteins. These results therefore have immediate implications for the effective design of blood-based biomarker studies.

Keywords heritability; longitudinal variability; plasma biomarkers; SWATH-MS; twin study

Subject Categories Genome-Scale & Integrative Biology; Genetics, Gene Therapy & Genetic Disease; Post-translational Modifications, Proteolysis & Proteomics

DOI 10.15252/msb.20145728 | Received 27 August 2014 | Revised 7 January 2015 | Accepted 12 January 2015


Introduction

The effects of genomic variation, modulated by lifestyle and environment, orchestrate the extensive phenotypic variability found in human populations. The quantification of narrow-sense heritability, that is, the proportion of phenotypic variance attributable to additive genetic effects, provides important information for basic and disease biology (Lichtenstein et al., 2000; Stranger et al., 2007; Emilsson et al., 2008; Visscher et al., 2008). Within the human population, the narrow-sense heritability of traits can be determined in twin cohort studies. Monozygotic (MZ) twins are genetically identical and thus provide a natural and extremely valuable opportunity to estimate the relative importance of genes and environment by benchmarking MZ phenotype discordances to those of dizygotic (DZ) twins which, on average, share one half of the identical by descent genetic variability (Martin et al., 1997).

To date, such studies have been performed at the organismal phenotype, transcript (Grundberg et al., 2012; Wright et al., 2014), epigenetic (Grundberg et al., 2013) and metabolic levels (Nicholson et al., 2011; Shin et al., 2014), respectively. Proteins predominantly determine the biochemical state of biological specimens and proteomic variation is therefore thought to be closely associated with phenotypic variation, adding a complementary component to the corresponding nucleic acid-based indicators (Aebersold et al., 2005; Picotti et al., 2013; Wu et al., 2013). To date, the reproducible and quantitatively accurate mass spectrometric measurement of proteins across longitudinally collected proteome samples from MZ and DZ pairs of a twin cohort study has been technically challenging due to the high complexity and large dynamic range of human proteome samples, particularly the plasma proteome.

Human blood plasma is the prime source of protein biomarkers and one of the most intensely studied clinical specimens because it can be obtained by minimally invasive methods and contains protein biomarkers that indicate physiological and pathological changes associated with disease (Zhang et al., 2007). However, in
Published online: February 4, 2015

Molecular Systems Biology

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Despite an enormous number of studies, the clinically important properties of the plasma proteome remain largely unexplored. Specifically, the variability of plasma protein levels in a population, the heritability of protein levels and their longitudinal stability over time remain largely unexplored. Previous relevant population-based proteomic studies have either not been focused on the plasma proteome (Wu et al., 2013), or had limited analytical depth (Melzer et al., 2008; Kato et al., 2011; Lourdusamy et al., 2012; Johansson et al., 2013) [from 10s to 163 plasma proteins (Johansson et al., 2013)] or analytical preference (Enroth et al., 2014) and/or limited quantification robustness and outcome reproducibility (Melzer et al., 2008; Kato et al., 2011).

Here, we used SWATH-MS, an emerging high-throughput target-centric mass spectrometry method (Gillett et al., 2012; Liu et al., 2014; Rost et al., 2014), to quantify 342 plasma proteins across 232 plasma samples that were collected with 2- to 7-year intervals from MZ and DZ twin pairs. SWATH-MS essentially combines the analytic throughput of the traditional shotgun or discovery proteomics with the exquisite quantitative accuracy and reproducibility of selected reaction monitoring (SRM), the prototypical quantitative mass spectrometry technique. The data indicate that inherent variability of protein levels varies significantly for different plasma proteins and that the regulation of specific protein levels and biological processes is under tight genetic and/or temporal control. To the best of our knowledge, this is the first study that applies the current, quantitatively accurate mass spectrometric approaches to a twin study for analyzing protein heritability determinants in the (clinically relevant) plasma samples with the unique design of longitudinal sampling.

Results

The reproducible quantification of plasma proteins across a longitudinal twin cohort by SWATH-MS

To quantify the levels of human plasma proteins, we applied the newly developed SWATH-MS technique (Gillett et al., 2012) in a longitudinal twin cohort. The cohort consisted of 44 DZ and 72 MZ twins from the Twins UK Adult Twin Registry where blood samples were drawn at two different time points (Fig 1). Twins were selected at an average age of 57.8 years at the first visit, ranging from 38 to 74 years of age. The time interval between the two samplings was 5.2 ± 1.4 years. The twins had an average age of 63.1 at the time of second visit, ranging from 44 to 78 years of age (see Supplementary Fig S1 for the distribution of actual age in the cohort at the two visits). Fasted plasma samples were collected at identical conditions (see Materials and Methods and Supplementary Table S1). Females were chosen to simplify the experimental design by excluding the gender variance.

The data-independent acquisition (DIA) mass spectrometric quantification method of SWATH-MS essentially converts all the peptides ionized from a biological sample into a high-resolution, digital map of fragment ion signals (Gillett et al., 2012; Liu et al., 2013a) (Fig 1). In these maps, specific proteins were monitored via a targeted data analysis strategy, where fragment ion signal groups uniquely identifying a targeted peptide were detected and quantified in each of the 232 SWATH-MS maps. The parameters of the signal group for each peptide, including the fragment ion masses, their relative intensity and chromatographic concordance, the peptide retention time and precursor mass range, constituted a specific assay for each targeted peptide that was prepared a priori from a spectral library of the human plasma proteome (Fig 2A). Specifically, to generate this spectral library, we deployed comprehensive shotgun proteomic sequencing of the plasma digest of a mixed plasma sample, which was firstly depleted of the 14 most abundant proteins and then fractionated by strong anion exchanger at the peptide level, yielding specific assays for 652 proteins. Further, we included in the library additional MS assays for plasma proteins (Farrah et al., 2011) from an in-house SWATH assay compendium for the human proteome (Rosenberger et al., 2014). The final combined library contained more than 43,000 peptides, representing 1,667 unique plasma proteins, which represents, as of to date, the largest SWATH-ready spectral library for the human plasma proteome (freely provided with raw data), and therefore maximized the number of identified proteins from the SWATH maps.

Using this library and the OpenSWATH software framework (Rost et al., 2014), we confidently identified 4,271 unique peptides at an FDR of 1%, corresponding to 534 distinct proteins in all the unfractionated and non-enriched plasma samples (Fig 2A and Supplementary Table S2). Their levels in plasma were estimated to cover six orders of magnitude according to human plasma Peptide-Atlas database (Farrah et al., 2011), reaching, for some proteins, to levels as low as several nanograms per milliliter (Supplementary Fig S2A). On average, 3,520 peptides and 425 proteins were identified from each twin sample. We further filtered these data to select the 1,904 peptides that unambiguously associated with 342 UniProt proteins (Mallick et al., 2007) and that were consistently quantified among samples. These peptides constituted a data matrix with only 10.07% missing values across 232 samples and approached 0% missing values after applying the abundance re-quantification algorithm of OpenSWATH (Rost et al., 2014) (see Materials and Methods). The dataset contained 42 (that is, about 40%) of protein biomarker analytes whose measurement has been approved by US Food and Drug Administration (FDA) for clinical purpose (hereafter, clinically assayed proteins) assayed in blood (Anderson, 2010). It compares favorably to prior multisample human plasma studies regarding analytical depth (Melzer et al., 2008; Kato et al., 2011; Lourdusamy et al., 2012; Johansson et al., 2013), particularly considering that the analytical time was a mere 2.5 h per sample and consumed only 0.015 µl of plasma per SWATH injection, and significantly exceeds the previous studies in terms of reproducibility and quantitative accuracy.

We next sought to assess the properties of the SWATH data. First, we calculated the coefficient of variance (CV) of protein level for each protein. Overall, 84.5 and 76.0% of the proteins quantified in technical and whole-process experimental replicates had CVs of < 25%. The median CV for technical replicates was 7.2% (Fig 2B) and for whole-process experimental replicates 14.2%. Second, we compared the quantitative data of SWATH-MS to SRM measurements where we spiked the samples with heavy stable isotope-labeled reference peptides and performed SRM analyses using established SRM assays (Huttenhain et al., 2012). As expected, the ratios between light and heavy versions of 41 peptides detected in all the 232 SWATH maps were generally well correlated with SRM results among the samples, with a mean of $R = 0.85$ (Supplementary Fig S2B and C). Third, we performed unsupervised hierarchical clustering analysis (HCA) among SWATH maps (Fig 2C). HCA found
that 137 (i.e. 59.1% of 232) plasma samples from either the twin pairs or longitudinally sampled individuals (96 samples from 144 MZ twins and 41 from 88 DZ twins) were directly clustered as adjacent nodes, indicating the global proteomic similarity between these samples.

Substantial variability in plasma protein levels was observed among individuals. Overall, 174 (i.e. 50.8% of 342) proteins showed more than tenfold change of SWATH signals between the extremes of the entire cohort. The standard deviation of the fold change of one protein intensity from its average level ranged from 0.1403 for antithrombin III and 0.1465 for vitamin D-binding protein to 1.1936 for apolipoprotein(a) and 1.6871 for serum amyloid A-1 protein, respectively (Fig 2D), indicating that protein-level variability is an important feature for different plasma proteins within human population.

Variance decomposition in the quantitative human plasma protein dataset

We took advantage of the longitudinal twin design and utilized a linear mixed model (Nicholson et al., 2011) to systematically partition the variance observed for 342 protein levels. The observed overall variance of protein abundance was partitioned into four biological contributing factors (heritable, common environmental, individual environmental and longitudinally contributing factors) and unexplained effects using a linear mixed model.
“family component”, which explains almost half (i.e. 46.2%) of the biological variance in our results. Table 1 lists the five proteins most strongly affected by each biological component (see Supplementary Table S3 for variance decomposition results of all proteins). For example, the plasma level of apolipoprotein(A) was determined to be the most strongly heritable ($h^2 = 0.6633$), a finding that is consistent with previous reports (Boerwinkle et al., 1992; Lopez et al., 2008; Cenarro et al., 2014).

We checked if possible modifications of peptides of the identified proteins could affect the variance decomposition results. We found that only < 5% of the peptides included in our model as protein quantitative evidence could be possibly modified according to the Swiss-Prot database with any type modification site reported in the human proteome (Supplementary Table S2 and Supplementary Fig S3A). Even if only these possibly modified peptides were included in the analysis, identical results to those mapped proteins

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Figure 2. Proteomic identification and reproducible quantification among the twin cohort.
A The establishment of a comprehensive, specific spectral library of human plasma proteome that is ready for SWATH-MS analysis. The reference spectra were generated a priori by extensive shotgun proteomic sequencing of fractionated plasma peptides and complemented with spectra of additional known plasma proteins (Farrah et al., 2011).
B The coefficient of variance (CV) analysis at technical, whole-process experimental and cohort levels.
C Heatmap of hierarchical clustering analysis of the protein vs. sample matrix indicates that plasma proteins were reproducibly quantified by SWATH-MS across the sample cohort.
D The distribution of the fold changes of protein SWATH-MS intensities from their average abundance levels across the cohort is shown as box plots. Antithrombin III (ANT3) and vitamin D-binding protein (VTDB) are shown as examples of the most stable proteins, whereas apolipoprotein(a) (APOA) and serum amyloid A-1 protein (SAA1) are shown as examples of the most variable proteins. The red bars show the positions of the protein analytes whose measurement has been cleared or approved by FDA in human blood.
were achieved with respect to determining the contribution of family components to the total variance ($R = 0.83$, Supplementary Fig S3B). This analysis thus suggests that the peptides included in our model for protein quantification are dominated by their naked forms and the peptide-level modification status has an undetectable and negligible effect on our results.

We then compared our results to the only previous twin study of the similar design, in which 58 plasma proteins were analyzed by using also a female twin cohort from Twins UK but an antibody bead-based technology (Kato et al., 2011). The SWATH-MS yielded heritability values for an additional 284 proteins, extending the scope of the study by a factor of six. Most importantly, compared to the antibody technique applied, SWATH-MS achieved a higher degree of reproducibility, obviating the need to remove any outlying samples (Kato et al., 2011) (Supplementary Fig S4A–D) and translating into a significantly higher fraction of total phenotype variance that can be explained by biologically stable factors ($P = 5.19e-7$, Wilcoxon rank-sum test, Supplementary Fig S4E–G).

Intriguingly, in our result the longitudinal factor could explain 13.6% of the phenotype variance, compared to 2.9% reported by Kato et al (2011) where the same conceptual variance model as that of our study was used. This discrepancy may be mainly ascribed to the much shorter temporal intervals of sampling used in their study (around 3 months), indicating that the natural aging process together with other longitudinally unstable factors during the ~5-year period tested in the present study in total uncovered a profound impact of a relatively long-term, temporal changes on human plasma proteomic dynamics. We also carefully checked the existence of other longitudinal factors besides an aging effect (Supplementary Table S1). We found two individuals (i.e. 1.7% of 116 twins) in the cohort who developed cancer between the two visits, and at least 6.9–17.2% of the samples had changed menopausal status at the time of the two visits. A total of 15 (i.e. 12.9% of 116) twins had confirmed type II diabetes before visit one. No individual developed new diabetes type II at visit 2 in this cohort. According to the usage of four types of common medications (corticosteroids, thyroxine, statins and antihypertensives), we found that

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**Figure 3. Dissection of the plasma protein-level variability.**

A Histograms of contribution percentage of each biological component (red: heritability; light blue: common environment; dark blue: individual environment; yellow: longitudinal effects; gray: unexplained fraction) determined by a linear mixed model based on the longitudinal twin design. Selected protein names are shown for those clinically assayed proteins with the most heritable levels, for example, APOA, plasma protease C1 inhibitor (IC1), complement C4-A (CO4A), fibrinogen alpha chain (FIBA) and pancreatic triacylglycerol lipase (LIPP), and for the most variable proteins among the cohort, for example, SAA1, APOA, pregnancy zone protein (PZP), trifunctional enzyme subunit alpha (ECHA) and C-reactive protein (CRP).

B Examples of protein abundance correlations between MZ and DZ pairs. Fibrinogen beta chain (FIBB), Serum paraoxonase/arylesterase 1 (PON1).
Table 1. Top proteins mostly affected by the four biological variance components

<table>
<thead>
<tr>
<th>Swiss-Prot I.D.</th>
<th>Protein name</th>
<th>Gene name</th>
<th>Heritability</th>
<th>Common environment</th>
<th>Individual environment</th>
<th>Longitudinal effects</th>
<th>Unexplained</th>
<th>Direct literature support</th>
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<tbody>
<tr>
<td>P08191</td>
<td>Apolipoprotein(a)</td>
<td>LPA</td>
<td><strong>0.6633</strong>*(0.3734–0.9538)</td>
<td>0.1845</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.1521</td>
<td>Yes</td>
</tr>
<tr>
<td>P04220</td>
<td>Ig mu heavy chain disease protein</td>
<td>-</td>
<td><strong>0.6542</strong>*(0.5297–0.7787)</td>
<td>0.0000</td>
<td>0.1382</td>
<td>0.0000</td>
<td>0.2077</td>
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<tr>
<td>Q04366</td>
<td>CDS-antigen-like</td>
<td>CDSL</td>
<td><strong>0.6180</strong>*(0.2952–0.9408)</td>
<td>0.0409</td>
<td>0.1877</td>
<td>0.0000</td>
<td>0.1534</td>
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<tr>
<td>Q03591</td>
<td>Complement factor H-related protein 1</td>
<td>CFHR1</td>
<td><strong>0.6149</strong>*(0.4902–0.7396)</td>
<td>0.0000</td>
<td>0.0403</td>
<td>0.0099</td>
<td>0.3349</td>
<td>Yes</td>
</tr>
<tr>
<td>Q02985</td>
<td>Complement factor H-related protein 3</td>
<td>CFHR3</td>
<td><strong>0.6066</strong>*(0.4781–0.7352)</td>
<td>0.0000</td>
<td>0.0249</td>
<td>0.0000</td>
<td>0.3685</td>
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</tr>
<tr>
<td>P01877</td>
<td>Ig alpha-2 chain C region</td>
<td>IGH2</td>
<td>0.0194</td>
<td>0.6688</td>
<td>0.2222</td>
<td>0.0082</td>
<td>0.0813</td>
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<tr>
<td>P00748</td>
<td>Coagulation factor XII</td>
<td>F12</td>
<td>0.0000</td>
<td>0.5862</td>
<td>0.1755</td>
<td>0.0764</td>
<td>0.1619</td>
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<tr>
<td>P31939</td>
<td>Bifunctional purine biosynthesis protein PURH</td>
<td>ATIC</td>
<td>0.0000</td>
<td>0.5605</td>
<td>0.1253</td>
<td>0.0065</td>
<td>0.3078</td>
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<tr>
<td>P01860</td>
<td>Ig gamma-3 chain C region</td>
<td>IGH3</td>
<td>0.0000</td>
<td>0.5531</td>
<td>0.2229</td>
<td>0.0364</td>
<td>0.1876</td>
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<tr>
<td>O75639</td>
<td>Ficolin-3</td>
<td>FCN3</td>
<td>0.0000</td>
<td><strong>0.5059</strong></td>
<td><strong>0.1081</strong></td>
<td>0.0916</td>
<td>0.2944</td>
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<tr>
<td>Q99459</td>
<td>Cell division cycle S-like protein</td>
<td>CDC5L</td>
<td>0.0682</td>
<td>0.0000</td>
<td><strong>0.5295</strong></td>
<td>0.1457</td>
<td>0.2566</td>
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<tr>
<td>P01861</td>
<td>Ig gamma-4 chain C region</td>
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<td>0.0000</td>
<td>0.4276</td>
<td><strong>0.5185</strong></td>
<td>0.0045</td>
<td>0.0495</td>
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<tr>
<td>P01777</td>
<td>Ig heavy chain V-III region TE1</td>
<td>-</td>
<td>0.1278</td>
<td>0.0155</td>
<td><strong>0.3881</strong></td>
<td>0.0794</td>
<td>0.3892</td>
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<tr>
<td>P07419</td>
<td>Beta-2-glycoprotein 1</td>
<td>APOH</td>
<td>0.0000</td>
<td>0.1009</td>
<td><strong>0.3807</strong></td>
<td>0.1324</td>
<td>0.3861</td>
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<tr>
<td>P43450</td>
<td>Nicotinamide phosphoribosyltransferase</td>
<td>NAMPT</td>
<td>0.0010</td>
<td>0.0000</td>
<td><strong>0.3594</strong></td>
<td>0.2425</td>
<td>0.3970</td>
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<tr>
<td>Q562R1</td>
<td>Beta-actin-like protein 2</td>
<td>ACTBL2</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td><strong>0.3554</strong></td>
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<tr>
<td>P48681</td>
<td>Nestin</td>
<td>-</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0938</td>
<td><strong>0.4692</strong></td>
<td>0.4371</td>
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<tr>
<td>Q163531</td>
<td>DNA damage-binding protein 1</td>
<td>DDB1</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.1139</td>
<td><strong>0.4560</strong></td>
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<tr>
<td>P19667</td>
<td>Hexokinase-1</td>
<td>HK1</td>
<td>0.1918</td>
<td>0.0000</td>
<td>0.0000</td>
<td><strong>0.4510</strong></td>
<td>0.3572</td>
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<tr>
<td>P60174</td>
<td>Triosephosphate isomerase</td>
<td>TPII</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.1137</td>
<td><strong>0.4413</strong></td>
<td>0.4450</td>
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</table>

*Denotes 95% CIs for the heritability estimates (see Supplementary Table S3 for all the proteins). The bold values highlight the significant contribution to the quantitative variance from respective root cause.
the twins tended to take more medications at the second visit (an average of 0.38 medications per person at visit 1 versus 0.53 medications per person at visit 2, $P = 0.0125$). This is consistent with the report from Enroth et al (2014) who found a Spearman rho equals 0.29 for the correlation between age and number of medications. In summary, the longitudinal nature plus the twin structure of our sample allowed us to give a quantification of the main causes of variation in protein levels in plasma.

**Differential biological processes preferably regulated by heritability and other biological factors**

Statistically significant heritability was observed for 80 proteins (i.e. 23% of 342, $h^2 > 0.25$ or $P < 0.01$). This percentage is close to the result of Johansson et al (2013) who measured plasma samples in the parent–children context and thereby determined the abundance levels of 19% of the plasma peptides to be heritable. We confirmed the high heritability of protein level for 21 of the proteins discovered by Johansson et al (2013). Additionally, we determined 60 plasma proteins, the level of which was closely associated with longitudinal changes, 52 with familial environment and 47 with individual environment. Among these, 17 proteins appeared to be regulated by both familial and individual environments. To discern the biological processes associated with the four biological sources of variability, we annotated the protein lists by Gene Ontology (GO) and pathway enrichment analysis. This analysis identified several protein functional clusters that are significantly affected by either heritability, environment or the longitudinal effects (Fig 4A). For example, a cluster of immune response proteins, consisting of proteins related to the innate immune response and inflammatory regulation ($P$-values between $P = 0.00032$ and $P = 2.60e-6$ for the enrichment significance in all relevant functional processes), the blood coagulation cluster ($P$-values between $P = 0.035$ and $P = 0.00019$) and a protein-processing cluster ($P$-values between $P = 0.040$ and $P = 1.33e-6$), were found to be more strongly heritable or familial than associating with individual environment and aging factors. Moreover, the clusters of proteins related to body fluid regulation ($P$-values between $P = 0.053$ and $P = 1.16e-5$), lipid metabolism ($P$-values between $P = 0.065$ and $P = 0.00050$) and protein secretion ($P$-values between $P = 0.021$ and $P = 1.53e-12$) were found to be not only heritable but also heavily interacting with individual environment. Interestingly, the functional cluster of hormone response was under tight regulation of the longitudinal effects ($P$-values between $P = 0.030$ and $P = 0.016$). These results are consistent with and extend previous literature reports. For example, Souto et al (2000) showed that the blood coagulation and fibrinolysis pathways are strongly determined by genetic factors in Spanish families, and Snieder et al (1999) noted the importance of genetic dependency of lipid system. Taken together, the twin proteomic data reveal that different biological processes are regulated by genetic control, and environmental or longitudinal factors to different degrees.

**The biological variance dissected for proteins of different plasma concentrations**

The systematic dissection of the origins of variance of plasma proteins may provide opportunities for new biological insights. For example, using the estimated concentration levels of plasma proteins from PeptideAtlas (Farrah et al, 2011), we investigated if the total variability (represented by standard deviations of the fold change of protein signals relative to the average level) and the relative contributions of the four biological components globally depended on the plasma concentration. The result suggests that most of low abundant proteins are more variable; however, there are a few high abundant proteins whose levels were also highly variable among the cohort, which in turn makes the statistical correlation between variability and protein concentration insignificant (Supplementary Fig S5). Importantly, by analyzing the relative contributions of factors explaining total biological variance, we found that the correlation between plasma protein levels and heritability values was low ($R = 0.087$, $P = 0.178$ for the correlation, Fig 4B), suggesting that the concentration levels of more abundant proteins are not necessarily more strongly determined by genes. The variability from both common and individual environmental components has no detectable associations with the protein concentrations. In contrast, concentration variability of more abundant proteins is generally less affected by longitudinal factors such as aging ($P = 0.0165$, Fig 4B). This novel finding could thus indicate the limited regulation power of, for example, longitudinal aging processes on highly abundant plasma proteins or that the longitudinal factors preferably control the levels of low abundant proteins.

**The heritability of circulating levels of high-density lipoproteins and other protein classes**

We next analyzed the source of variation in a clinically and biologically related set of proteins, those associated with high-density lipoproteins (HDL) (Shah et al, 2013). HDL represents a complex, bioactive particle in human plasma that can be isolated by density gradient ultracentrifugation and has been shown to minimally consist of 85 proteins (Shah et al, 2013). HDL has a range of roles, such as the promotion of reverse cholesterol transport and modulation of inflammation. Here, we quantified the circulating levels of 92% of putative HDL proteins (that is, 78 out of 85). The data indicate that HDL proteins showed a markedly higher heritability compared to the non-HDL protein quantified in this study ($P = 0.00011$, Fig 4C), suggesting that the biological roles (e.g. cardioprotective effect) of HDL in humans might be under high genetic control (Hegele, 2009).

We repeated the heritability analysis for proteins containing different types of modifications according to human Swiss-Prot database and different domains according to Pfam database. Interestingly, proteins annotated as “Glycoproteins” (Fig 4D, $P = 0.00071$) and as containing “Disulfide bond” were more strongly regulated by genetics and less affected by longitudinal factors compared to other proteins, while the proteins denominated “Phosphoprotein” or containing “Acetylation” showed a reversed regulation trend (Supplementary Fig S6, $P < 0.01$ or $P < 0.05$). We further found that proteins with “V-set domains” seemed to be highly regulated by family component (the combination of heritability and shared environment). These observations might be associated with the protein class functions; for example, proteins with V-set domain in blood are mainly immune proteins that are more strongly heritable (Fig 4A). Furthermore, considering the overlapping
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indeed harbor diverse genetic or longitudinal regulation to conclude whether different protein modifications or structures in the current study, we suggest that further direct studies are crucial to directly measure any protein modification and structure in the proteins between annotation classes and the fact that we did not determine the contribution in total heritability control of plasma protein concentration levels varied over time, we firstly separated the dataset according to the two longitudinal time points. To study the question whether the genetic contribution to the control of plasma protein concentration levels varied over time, we firstly separated the dataset according to the two longitudinal time points.

proteins between annotation classes and the fact that we did not directly measure any protein modification and structure in the current study, we suggest that further direct studies are crucial to conclude whether different protein modifications or structures indeed harbor diverse genetic or longitudinal regulation dependency.

Genetic contribution to plasma protein-level control between the two longitudinal visits

To study the question whether the genetic contribution to the control of plasma protein concentration levels varied over time, we firstly separated the dataset according to the two longitudinal time points.

Data information: P-values are determined by Wilcoxon rank-sum test.
points which were on average 5 years apart and then compared the heritability values of all the proteins quantified at the two sampling points. The results indicate (Fig 4E) that heritability generally decreased over time during the 5-year process, for the proteins tested ($P = 4.90e-07$). To corroborate this result, we included all the informative peptides of our proteins (instead of top abundant ones) for the statistical test and obtained the same global trend for majority of the peptides ($P < 2.2e-16$ based on 1,904 peptides, Supplementary Fig S7A). We then added together the heritability and the common environment factor as family component and observed that the family component still had a decreased trend of contribution to the protein variability between two time points ($P = 0.0008$, Supplementary Fig S7B). To further investigate this phenomenon, we factored the real age of each individual into the model and observed that the real age had a minimal effect in determining the heritability (correlation of 99%) and in explaining the variance (only explained 0–1% of the variance for most proteins) (Supplementary Fig S7C–H). It is worth mentioning that the real age could consistently explain >4% of the total variance for only two proteins, fibrinogen beta chain (FIBB) and lumican (LUM), at both visits. Altogether, these results suggest that in these relatively old individuals tested (average age of 57.8 at visit 1), the genetic regulation of many (but not all) plasma proteins decreased along with longitudinal processes or that the environmental factors and other factors unexplained by the linear mixed model have an increased contribution in proteomic variability during the 5-year temporal process.

**Insight for biomarker discovery studies derived by protein variability analysis**

We next interrogated the plasma-level variability for the 42 proteins whose clinical assays have been approved by FDA (Anderson, 2010) and that were quantified in the dataset. We observed that generally, their overall variability was lower than that of the other proteins across the cohort ($P = 0.0001$, Figs 4F and 2D). We partially ascribe the lower variability of clinically assayed proteins to their higher heritability ($P = 0.0133$, Supplementary Fig S8) and to the fact that it is desirable for biomarkers to have relatively stable expression levels. Strikingly, we found that the plasma levels of a few reported biomarkers are strongly and preferably affected by a longitudinal effect (>30%) compared to the heritability and environmental components (Fig 4G). These include biomarkers whose measurements have been already cleared or approved by FDA (e.g. biotinidase (Kang et al, 2010), platelet basic protein, thyroxine-binding globulin, etc.) and candidate proteins not yet cleared. For example, triosephosphate isomerase (TPIS) was discovered as a promising blood biomarker for metastatic non-small cell lung cancer (Patel et al, 2011), especially lung squamous cell carcinoma (Zhang et al, 2009). 14-3-3 protein zeta/delta (1433Z) was discovered as a putative prognostic marker for renal cell carcinoma (Masui et al, 2013) and for monitoring chemotherapy in breast cancer (Hodgkinson et al, 2012). And plasma level of soluble CD14 was indicated to be an independent predictor for human immunodeficiency virus infection. Our data therefore indicate that for the application of these proteins as predictive biomarkers, the longitudinal factors (such as aging) dependent variability should be considered. The incorporation of variability over time with stochastic variability of protein biomarkers will enable the refinement of risk scores estimated by such biomarkers.

**Identification of cis-protein quantitative trait loci (pQTL) influencing plasma protein levels**

Finally, we carried out association analysis to identify cis-SNPs regulating the levels of 303 (of the 342 measured) proteins that we could uniquely map to known genes. We first selected a total of 113 out of 116 individuals which passed the genotyping data quality control step. Second, we used the twin proteomic data to map the protein quantitative trait loci, or pQTLs by determining the statistical significance of the association between SNPs 1 Kb up and down the transcription start site for each gene and protein expression values. The final number of tested SNPs was 758. We found 13 genes with at least a statistically significant pQTL (Fig 5A). Among them, four plasma proteins (ficolin-2, coagulation factor XII, complement component C8 gamma chain and complement C5) were annotated with the “innate immune response” process. The close association between genotype alleles and protein levels is shown in Fig 5B. Similar distributions were obtained for all identified pQTLs (Supplementary Fig S9). We observed that most of the discovered pQTLs lie in regulatory regions and only 2 of them are in the coding region, but synonymous (Supplementary Table S4). To explore the functional role of the pQTLs, we assessed whether they have an effect on gene expression. We checked the association of the pQTLs with gene expression in four tissues (fat, skin, blood and lymphocyte cell lines (LCL)) in a cohort of about 800 female twins of the same population that are part of the Twin UK cohort with an identical age range. We called gene expression QTLs (eQTLs) in the four tissues by using gene expression measured by RNA-seq and genotype information (Buil et al, 2015). We could measure the gene expression of 9 of the 13 proteins with pQTLs, and we found that 5 of the 9 pQTLs are associated with eQTLs in at least one of the four tissues (Fig 5A and Supplementary Table S4).

We also compared our pQTLs to those published previously by Johansson et al (2013) which were not significant in our sample. This fact might be partially explained by the distinctive sample cohorts used. To further investigate if the difference in detection was just a matter of power, we checked at the $P$-values of Johansson’s pQTLs in our study. If the Johansson’s pQTLs were present in our sample, even if they were not statistically significant, we would expect to see an enrichment of small $P$-values in our sample. We estimated the mean of the $P$-value of the Johansson’s pQTL associations in our sample, and compared this mean value with the distribution of means expected if there were no signal in our sample. Supplementary Fig S10 shows the expected distribution of mean values under the null hypothesis (no signal of Johansson’s pQTLs in our sample) and the actual observed mean. From there, we calculated a $P$-value equals 0.0035 that supports the notion that with a larger sample, we would find some of the pQTLs described by Johansson et al (2013).

To estimate the relative contribution of the pQTLs to protein variability, we estimated the proportion of protein variance explained by each pQTL. We observed that these pQTLs explained between 3 and 19% of the protein’s variance with an average of about 8.5%.
To compensate the fact that heritability might be not well separated with the estimates of common environment, we then estimated the contribution of the pQTL to the total family component consisting of both heritability and common environment component. We observed that pQTLs explain between 6 and 68% of the family component, with an average of about 25% (Supplementary Table S5). Finally, we used the online annotation tool SnpNexus (Dayem Ullah et al., 2012) to annotate the pQTLs as disease SNPs. We found that two of the pQTLs are associated with the following diseases: rs1801020 on gene F12 with cardiovascular disease (Santamaria et al., 2004; Cochery-Nouvellon et al., 2007) and rs2071042 on gene ITIH4 with hypercholesterolemia (Fujita et al., 2004). In summary, we found pQTLs for 13 proteins that explained about 8.5% of the protein-level variance.

Discussion

The variability of human proteins within a population lays a basal-level foundation for relating protein expression patterns to disease (Nedelkov et al., 2005). The knowledge of the protein variability therefore stands to have an immediate impact in the field of clinical proteomics. In this study, we present the first study that addresses the degree of human protein variability and its origins using a quantitatively accurate proteomic approach and the rigorously controlled biological samples. Specifically, we used SWATH-MS (Gillet et al., 2012) to robustly and systematically dissect the generic and genetic variance within a cohort of 232 healthy twins by focusing on the proteome of the most intensely studied clinical samples, the human plasma. This analysis has become possible by the development of new mass spectrometric techniques that generate quantitatively accurate and highly reproducible datasets across large sample cohorts. Herein, by combining the extensive shotgun analysis presented in this study and the recently published SWATH-MS assay compendium for the human proteome (Rosenberger et al., 2014), we also configured the to-date most comprehensive spectral library for quantifying 1,667 human plasma proteins. We now provide this high-quality spectral library as a transferrable resource deposited in ProteomeXchange, which can be easily downloaded as a stand-alone MS assay repository to support those future targeted proteomic studies focused on the human plasma. Using this library and a targeted data analysis strategy, we identified on average 425 plasma proteins in each individual. This number is 3.3 times higher than that reported in the previous large-scale plasma proteomic...
survey by Johansson et al (2013). Further, in comparison to an extensive SRM analysis of body fluids that targeted more than 1,000 cancer-associated proteins (CAPs) and detected 73 proteins in the crude plasma digest (Huttenhain et al, 2012), herein we identified 99 CAPs and these were quantified across the whole twin cohort. Besides the high proteome coverage achieved, we consistently quantified 342 unique plasma proteins (assigned by 1,904 informative peptides) among the whole cohort with a highly completed data matrix facilitated by OpenSWATH framework (Rost et al, 2014).

Most importantly, the technical variability of our SWATH-MS profiling was indeed extremely low (an average CV of 7.2%), which, in essence, significantly reduced the technical variance and allowed for the rigorous decomposition of biological variances that can be relatively small.

In our longitudinal twin strategy, we used samples from twins at an older age (an average of 57.8 and 63.1 at two donations) for the practical consideration that many systematic diseases such as cancer occur more frequently at this age range. For example, over half (53%) of all cancers are diagnosed in adults aged 50–74 and over a third (36%) of all cancers are diagnosed in the elderly aged > 75 in UK, 2009–2011. Our biological variability analysis at this similar age range is thus beneficial to the further understanding of the plasma protein profiles reported from those biomarker discovery studies focusing on such diseases. Females were chosen to simplify the experimental design by excluding the gender variance component in the dissection model due to the limited sample size.

The same trait measured over an individual’s lifetime can have different genetic effects influencing it (Vischer et al, 2008). Therefore, compared to a parent–children-based approach (Johansson et al, 2013) which normally needs to remove the aging effect as a confounding factor, twin studies based on the longitudinal sampling of the same individuals are preferable to uncover the traits specifically regulated during a temporal period. We herein employed relatively longer longitudinal intervals of sampling, so that the controlling mechanism of plasma dynamics along with aging process during an average of 5.2 years could be investigated. We found the 5-year span was long enough to cause significant quantitative variability for 17.5% (that is, 60 out of 342) of plasma proteins. For slowly progressing diseases such as prostate cancer, 5 years might be not long enough to reveal the whole disease process. However, for many other general diseases, a span of 5 years is clinically relevant. For example, for ovarian cancer, the serum biomarkers were reported to provide evidence of the cancer 3 years before clinical diagnosis (Anderson et al, 2010). Also for diabetic nephropathy, the urinary levels of collagen fragments were demonstrated as prominent biomarkers 3–5 years before onset of macro-albuminuria (Zurbig et al, 2012). Previously, a 4-year difference in long-term storage was tested with no effect on plasma protein levels (Mitchell et al, 2005). This is consistent with our result where decreased genetic control along with the 5-year span can be successfully revealed. The biological confounding factors along with the 5-year span could include cancer, diabetes and other diseases developed, a change in medications consumed and menopausal status in elder women, as listed in Supplementary Table S1 as well as other temporal factors of unknown origin. With the limited cohort size, we could not dissect the variance contributed by these factors from the aging process in the present study. Of note, the relative longitudinal process explained much more variance than the real age of each individual (Supplementary Fig S7), demonstrating the necessity of the longitudinal strategy applied on the same individuals. With higher sample throughput rendered by the fast-developing proteomic instruments and workflows, a further, larger scale twin survey involving multiple longitudinal sampling points, a longer term age span that extends the age range investigated and both genders, would be ideal in the future to reinforce the understanding of genetic and longitudinal regulations of plasma protein expressions.

Additionally, the pQTLs we observed in the cohort may explain just a fraction of the genetic effects that control protein level. Other genetic variants in the cis-region around the functional gene plus genetic variants in trans-, far from the gene, are expected to explain the remainder of the observed heritability. The identification of these variants would require much larger sample sizes.

Moreover, we did not adopt the affinity depletion or enrichment approaches (which may bring more protein identifications) except for the step of spectral library generation, so that protein-dependent technical variation between individuals in the sample preparation step can be minimized to increase the power for our proteomic variance investigations. For future comparative studies, the relative variability derived from this study for certain plasma proteins (e.g. those interacting with albumin) might need to be adjusted by factoring in the technical variations, for example, those from immunodepletion (Dayon et al, 2014) or protein isolation steps, if indeed these steps are used.

Until now, biomarker discovery studies have almost entirely been focused on the comparison of the protein levels between disease and normal cases and were severely limited in sample size. However, the inherent (in)stability of proteins is biologically associated with its genetic architecture and thus, resultant confounding factors might obscure the biomarker analyses. Here, we show that the roles of the heritable, environmental and longitudinal determinants in controlling plasma protein levels are different for different proteins and functional clusters, and we noted that longitudinal effects might decrease the genetic control of protein levels and reduce the regulation power in controlling the variability of high abundant plasma proteins. All such valuable information will increase the ability of future studies to assign statistical significance to potential protein biomarkers.

Understanding the underlying genetic determinants of biomarkers can be useful in diagnosis or prognosis of diseases in two different ways. First, for biomarkers known to be causal for a disease, genetic variants associated with a biomarker become themselves genetic biomarkers for the disease. In this case, the quality of the genetic biomarker will depend on the association with the original biomarker. Second, for biomarkers that are not causal for a disease, the information about the disease that is carried by the biomarker does not come from itself but from unknown marker associated with, and causal for, the diseases. In this case, removing the effect of genetic variants that affect the biomarker abundance should increase the relative information of the unknown causal marker, making the biomarker more informative, that is, more associated with the disease. As examples, two identified plasma proteins, lipoprotein A (LPA) and C-reactive protein (CRP), were previously established as biomarkers for indicating coronary artery disease (CAD) risk (Jansen et al,
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Molecular Systems Biology

Published online: February 4, 2015

2014). LPA was reported as a causal risk factor for CAD (Jansen et al., 2014), and pQTLs for LPA are also associated with CAD (Kamstrup et al., 2009). That means that these pQTLs could be genetic biomarkers for CAD. On the other hand, pQTLs for CRP showed no association with CAD (Elliott et al., 2009); in this case, removing the effect of the genetic variants associated would result in a better scrutiny of CRP as a CAD biomarker.

In conclusion, the large-scale establishment of the heritability dimension of plasma proteins (or other subproteomes of human, or even the entire human proteome in the future) and the identification of pQTLs explaining a fraction of protein-level variation will sharpen our understanding of protein expressions, functions and temporal dynamics from the heritability perspective and aid the more efficient biomarker discovery. Our study serves as a first step in understanding the population-level variance components and eventually incorporating them in disease risk assessment.

Materials and Methods

Sample recruitment and collection

A total of 116 twins, comprising 22 DZ and 36 MZ pairs, were ascertained from the Twins UK Adult Twin Registry at King’s College London of approximately 11,000 twins (http://www.twinsUK.ac.uk) (Moayyeri et al., 2013) and invited to participate in this study. Eligible twins were healthy, Caucasian females of north European descent and aged between 38 and 78 years. They provided written informed consent. Zygosity was confirmed by genotype. We applied a longitudinal twin strategy to recruit samples. These healthy twins were selected at an average age of 57.8, and the time intervals between two donations range from 678 to 2,718 days, with a mean of 1,910 days (i.e. 5.2 years). This study was approved by St. Thomas’ Hospital Research Ethics Committee. The twins have no apparent history of cardiovascular disease (Buist, 2009); in this case, plasma samples were randomly collected from all the twins was firstly depleted of the 14 most abundant proteins with the multiple affinity removal system (MARS Hu-14 spin cartridge; Agilent Technologies) according to the manufacturer’s instruction. Depleted samples were exchanged with Vivaspyn 500 concentrators with a 5,000 molecular weight cutoff (Sartorius Stedim Biotech), denatured in 6 M urea and then diluted and digested with trypsin as above (Huttenhain et al., 2012). 50 µg of the resulting peptides was then separated into six fractions by strong anion exchanger (SAX) and purified on C18 StageTips as previously described (Wisniewski et al., 2009). The depleted sample was also directly digested and analyzed without SAX fractionation.

Sample preparation and protein digestion

Crude plasma samples were centrifuged at 18,400 g for 10 min at 4°C. The following sample preparation steps were performed with 96-well format plates with five whole-process experimental replicates distributed in different plates. 5 µl of plasma from each sample was diluted to 50 µl and filtered by G-10 gel filtration cartridges (Nest Group Inc.). Three external proteins were spiked (bovine alpha-1-acid glycoprotein with the targeted plasma level at 85 µg/ml, bovine fetuin-B at 8.5 µg/ml and human prostate-specific antigen at 0.85 µg/ml) before 80 µl of 10 M urea in 100 mM ammonium bicarbonate was added into each sample for denaturing at 37°C, 30 min. After reduction and alkylation with 10 mM tris(carboxyethyl)phosphine (Sigma-Aldrich) and 20 mM iodoacetamide (Sigma-Aldrich), the samples were diluted to 1 M urea and were digested with sequencing-grade porcine trypsin (Promega) at a protease/protein ratio of 1:50 overnight at 37°C (Huttenhain et al., 2012). Digests were purified with Vydac C18 Silica MicroSpin columns (The Nest Group Inc.). An aliquot of retention time calibration peptides from iRT-Kit (Biognosys) was spiked into each sample at a ratio of 1:30 (v/v) before all LC-MS analysis, to correct relative retention time differences between runs (Escher et al., 2012). Selected, heavy isotope-labeled internal standard peptides according to our previous study were synthesized (Huttenhain et al., 2012) (JPT Peptide Technologies and Thermo Fisher) and spiked into each sample for SRM and SWATH-MS measurements.

Plasma depletion and SAX fractionation

For the comprehensive shotgun analysis, crude plasma mixture from all the samples was firstly depleted of the 14 most abundant proteins with the multiple affinity removal system (MARS Hu-14 spin cartridge; Agilent Technologies) according to the manufacturer’s instruction. Depleted samples were exchanged with Vivaspyn 500 concentrators with a 5,000 molecular weight cutoff (Sartorius Stedim Biotech), denatured in 6 M urea and then diluted and digested with trypsin as above (Huttenhain et al., 2012). 50 µg of the resulting peptides was then separated into six fractions by strong anion exchanger (SAX) and purified on C18 StageTips as previously described (Wisniewski et al., 2009). The depleted sample was also directly digested and analyzed without SAX fractionation.

Shotgun proteomic measurement

The depleted and fractionated peptides were all measured by an AB Sciex 5600 TripleTOF mass spectrometer operated in data-dependent acquisition (DDA) mode. The mass spectrometer was interfaced with an Eksigent NanoLC Ultra 2D Plus HPLC system as previously described (Gillet et al., 2012; Collins et al., 2013; Liu et al., 2013b). Peptides were directly injected onto a 20-cm PicoFrit emitter (New Objective, self-packed to 20 cm with Magic C18 AQ 3-µm 200-Å material) and then separated using a 120-min gradient from 2 to 35% (buffer A 0.1% (v/v) formic acid, 2% (v/v) acetonitrile, buffer B 0.1% (v/v) formic acid, 90% (v/v) acetonitrile) at a flow rate of 300 nL/min. MS1 spectra were collected in the range 360–1,460 m/z. The 20 most intense precursors with charge state 2–5 which exceeded 250 counts per second were selected for fragmentation, and MS2 spectra were collected in the range 50–2,000 m/z for 100 ms. The precursor ions were dynamically excluded from reselection for 20 s.

SWATH-MS measurement

SWATH-MS measurements were performed with peptide mixtures generated by digesting crude plasma samples. The unfraccionated, total peptide samples were analyzed to minimize confounding factors introduced by sample handling. The same LC-MS/MS system used for DDA measurements was used for SWATH analysis (Gillet et al., 2012; Collins et al., 2013; Liu et al., 2013b). Specifically, in SWATH-MS mode, the instrument was specifically tuned to optimize the quadrupole settings for the selection of 25-m/z wide precursor ion selection windows. Using an isolation width of
26 m/z (containing 1 m/z for the window overlap), a set of 32 overlapping windows was constructed, covering the precursor mass range of 400–1,200 m/z. The effective isolation windows can be considered as being 399.5–424.5, 424.5–449.5, etc. SWATH-MS2 spectra were collected from 100 to 2,000 m/z. The collision energy (CE) was optimized for each window according to the calculation for a charge 2+ ion centered upon the window with a spread of 15 eV. An accumulation time (dwell time) of 100 ms was used for all fragment ion scans in high-sensitivity mode, and for each SWATH-MS cycle, a survey scan in high-resolution mode was also acquired for 100 ms, resulting in a duty cycle of ~3.4 s. Six repeated SWATH-MS measurements were performed on one of the samples to access the technical variability.

**SRM measurement**

For SRM analysis, peptide samples were analyzed on a hybrid triple quadrupole/ion trap mass spectrometer (5500QTRAP, AB Sciex) equipped with a nanoelectrospray ion source. Chromatographic separation of peptides was performed by a nanoLC ultra 1Dplus system (Eksigent) coupled to a 15-cm-fused silica emitter. Peptides were separated in a 35-min gradient of 5–35% acetonitrile in 0.1% formic acid (v/v) at a flow rate of 300 nl/min (Huttenhain et al., 2012; Liu et al., 2013b). Both Q1 and Q3 operated at unit resolution and a cycle time of 3 s at scheduled mode (4 min window). CEs were calculated according to previous studies (Lange et al., 2008; Liu et al., 2013b). SRM data were analyzed using Skyline (MacLean et al., 2010) and normalized based on the external proteins spiked and the heavy peptide standards.

**Spectral library generation for SWATH-MS**

Profile-mode.wiff files from shotgun data acquisition were centroided and converted to mzXML format using the AB Sciex Data Converter v.1.3 and converted to mzXML format using MSConvert v.3.04.238. The MS2 spectra were queried against the reviewed canonical Swiss-Prot complete proteome database for human (Nov. 2012) appended with common contaminants and reversed sequence decoys (Elias & Gygi, 2007) (40,951 protein sequences including decoys). The SEQUEST database search (Yates et al., 1995) through Sorcerer PE version 4.2 included the following criteria: static modifications of 57.02146 Da for cysteines and variable modifications of 15.99491 Da for methionine oxidations. The parent mass tolerance was set to be 50 p.p.m, and mono-isotopic fragment mass tolerance was 0.5 Da (which was further filtered to be < 0.05 Da for building spectral library); semi-tryptic peptides and peptides with up to two missed cleavages were allowed. The identified peptides were processed and analyzed through Trans-Proteomic Pipeline 4.5.2 (TPP) (Keller et al., 2005) and were validated using the PeptideProphet score (Keller et al., 2002). All the peptides were filtered at a false discovery rate (FDR) of 1%. The raw spectral libraries were generated from all valid peptide spectrum matches and then refined into the non-redundant consensus libraries (Collins et al., 2013) using SpectraST (Lam et al., 2007). For each peptide, the retention time was mapped into the iRT space (Escher et al., 2012) with reference to a linear calibration constructed for each shotgun run, as previously described (Collins et al., 2013). The MS assays, constructed from Top six most intense transitions with Q1 range from 400 to 1,200 m/z excluding the precursor SWATH window, were used for targeted data analysis of SWATH maps.

A published high-confidence list of 1.929 human plasma proteins compiled from PeptideAtlas (Farrah et al., 2011) was merged with an in-house compendium of MS assays for the targeted detection of any of more than 10,000 human proteins in SWATH-MS datasets (Rosenberger et al., 2014), and correspondingly, the highly specific MS assays of 1,550 plasma proteins were extracted. The spectral library from our shotgun analysis was then combined with the library of these 1,550 proteins at the peptide level, whereas the assays coming from the latter case were only accepted if the corresponding peptides were not identified by our shotgun effort.

**Targeted data analysis for SWATH maps**

SWATH-MS.wiff files were first converted to profile mzXML using ProteoWizard (Kessner et al., 2008). The whole process of SWATH-targeted data analysis was carried out using OpenSWATH (Rost et al., 2014) running on an internal computing cluster. OpenSWATH utilizes a target-decoy scoring system like mProphet to estimate the identification of FDR (Rost et al., 2014). The best scoring classifier that was built from the sample of most protein identifications was utilized in this study. Based on our final spectral library for human plasma proteome, OpenSWATH firstly identified the peak groups from all individual SWATH maps at a global peptide FDR = 1% (enabled by the strict FDR cutoff of 0.0307% at the level of total peak groups) and aligned them between SWATH maps based on the clustering behaviors of retention time in each run with a non-linear alignment algorithm (Weisser et al., 2013). Specifically, only those peptide peak groups identified in more than 1/3 samples were reported and considered for alignment with the max FDR quality of 0.1 (quality cutoff to still consider a feature for alignment) and/or the further constraint of < 100 s RT difference in LC gradient after iRT normalization.

Next, to obtain a high-quality quantitative data at the protein level, at the first step, we only accepted those proteins whose peptides had been identified in at least 90% of all the samples for proteomic profiling. Moreover, peptides that were shared between different proteins [non-proteotypic peptides (Mallick et al., 2007)] were discarded for quantification. The re-quantification option by OpenSWATH (Rost et al., 2014) was then enabled to re-quantify the missing values. Secondly, to retrieve the protein quantification information from those peptides identified in more than 1/3 but < 90% of the samples, we firstly enabled the re-quantification option of OpenSWATH to re-quantify the missing values by the local MS2 noise, then we fit our model at peptide-level, accepted those peptides whose residual variance (see below section of variance decomposition) was < 65% (so that an equal average of residual variance was achieved when compared to the first step). To quantify the protein abundance levels across 232 samples, we summed up the most abundant identified peptides (that is, 595 peptides) for each protein (Top three peptides, if > 3 peptides identified). This allows for reliably estimating global protein-level changes as shown in previous studies (Cima et al., 2011; Ludwig et al., 2012; Liu et al., 2013b, 2014; Weisser et al., 2013). The re-quantification in both steps totally retrieved signals for 10.07% of the missing cells in the protein-level data matrix. The two-step filtering strategy essentially discarded those peptides that were not detected in majority of the
samples (but got imputed by highly variable noisy background using re-quantification algorithm) and therefore filtered the consistent, high-quality protein signal groups among samples so that enough data points could be used for further variance decomposition. In total, quantification data of 342 unique Swiss-Prot proteins across all the 232 twin plasma samples were used for subsequent analyses.

Genotyping and imputation

Our samples are a subset of the Twins UK dataset. SNP genotyping of the Twins UK dataset (N = 6,000) was done with a combination of Illumina HumanHap300, HumanHap610Q and 1M-Duo chips and was performed by The Wellcome Trust Sanger Institute and National Eye Institute via NIH/CIDR. Similar exclusion criteria were applied to each of the three datasets separately. Sample exclusion criteria were (i) sample call rate < 98%, (ii) heterozygosity across all SNPs ≥ 2 SD from the sample mean, (iii) evidence of non-European ancestry as assessed by PCA comparison with HapMap3 populations and (iv) observed pairwise IBD probabilities suggestive of sample identity errors. We corrected misclassified monozygotic and dizygotic twins based on IBD probabilities. SNPs exclusion criteria were: (i) Hardy–Weinberg P-value < 10^{-6}, assessed in a set of unrelated samples; (ii) MAF < 1%, assessed in a set of unrelated samples; and (iii) SNP call rate < 97% (SNPs with MAF ≥ 5%) or < 99% (for 1% < MAF < 5%). Prior to merging, we performed pairwise comparison among the three datasets and further excluded SNPs and samples to avoid spurious genotyping effects, identified as follows: (i) concordance at duplicate samples < 1%; (ii) concordance at duplicate SNPs < 1%; (iii) visual inspection of QQ plots for logistic regression applied to all pairwise dataset comparisons; (iv) Hardy–Weinberg P-value < 10^{-6}, assessed in a set of unrelated samples; and (v) observed pairwise IBD probabilities suggestive of sample identity errors. We then merged the three datasets, keeping individuals typed at the largest number of SNPs when an individual was typed at two different arrays. Samples were genotyped on a combination of the HumanHap300, HumanHap610Q, 1M-Duo and 1.2MDuo 1M Illumina arrays. Samples were imputed into the 1,000 Genomes Phase 1 reference panel (data freeze, 10/11/2010) (Abecasis et al., 2012) using IMPUTE2 (Howie et al, 2009) and filtered [MAF < 0.01, IMPUTE info value < 0.8 and HWE (P < 1e-5)]. We ended with 3,552,580 SNPs in 113 of our 116 individuals.

Variance decomposition of protein and peptide level

It is known that variance components models are sensitive to traits that have a distribution different from normal. To get robust results, we transformed the protein quantifications using a rank normal transformation. To estimate the variance components of plasma protein level, we used a linear mixed model with two fixed effects and four random effects:

\[ y_i = \mu + \beta_1 \text{plate}_i + \beta_2 \text{time}_i + \gamma g_i + \epsilon_i + \text{id}_{i} + \text{w}_{i} + e_i \]

where plate and time are fixed effects representing the sample plate and the time interval between the two time points (time 0 in time point 1 and the actual time interval in time point 2) and the rest are random effects:

\[ \sigma^2_{\text{g}} \] is the polygenic effect.
\[ \sigma^2_{\epsilon} \] is the shared environment effect.
\[ \sigma^2_{\text{id}} \] is the effect the individual environment.
\[ \sigma^2_{\text{w}} \] is effect of the visit (longitudinal effects, or aging effect in this study).
\[ e \] are the residuals.

These data present a complex structure of correlation because we have related individuals measured twice. The expected correlation structure between a twin pair can be represented as:

\[ \Omega = 2\Phi \sigma^2_{\text{g}} + C \sigma^2_{\text{c}} + \text{ID} \sigma^2_{\text{id}} + W \sigma^2_{\text{w}} + 1 \sigma^2_{\epsilon} \]

where \( \sigma^2_{\text{g}} \) represent the variance of the X component: \( 2\Phi \) is the expected kinship coefficient based on the observed relationships—that is, 1 for MZ twins, \( \frac{1}{2} \) for DZ twins and 0 otherwise.
\( C \) is the shared environment matrix, 1 per all the twin pairs.
\( ID \) is the individual matrix, it takes into account that the same individual is measured in time point 1 and in time point 2. It is 1 for samples of the same individual in time points 1 and 2, and 0 otherwise.
\( W \) is the visit matrix, it takes into account the fact that the two sisters of a twin pair went together to the visit. It is 1 for the two sisters at each visits, 0 otherwise.
\( I \) is the identity matrix.

Heritability was defined as \( h^2 = \frac{\sigma^2_{\text{g}}}{\sigma^2_{\text{total}}} \) and the rest of the variance components were defined in the same way. The statistical analyses were carried out using SOLAR v 6.5.8 software package (Almasy & Blangero, 1998).

It is worth to note that, due to the limited samples size, it is difficult to separate perfectly the heritability and the common environmental effects components. The sum of these two components interpreted as a family effect, however, estimates more robustly (Kato et al., 2011; Nicholson et al., 2011).

pQTL determination

High-throughput experiments can generate batch effects that are difficult to control. In the transcriptomics field, it is widely acknowledged that removing the first principal components of the expression dataset removes unknown batch effects and increases the power to find eQTLs. We borrowed this technique, and before the pQTL analysis, we regressed out the first 10 principal components of the protein data. Since our data samples are twins, they are not independent observations and we need to take that into account in our models. We used the approach previously described (Aulchenko et al., 2007a) to keep the residuals of a mixed model that removed the effects of the family structure using the implementation in GenABEL R package (Aulchenko et al., 2007b). We then transformed those residuals using a rank normal transformation to avoid undesired outlier effects of the associations.

To identify pQTL associations, we performed a linear regression of the transformed residuals on all the SNPs in a 1-Kb window around the transcription start site for each gene and kept the best association per gene. To assign statistical significance of our associations, we run 20,000 permutations for each protein to estimate a null distribution. We shuffled the sample name in the protein data and repeated the association analysis keeping the best association for each permutation. Using this null distribution, we calculated an
empirical \( P \)-value per each protein. This approach is better than having a uniform threshold for all the proteins because we take into account that for some proteins, we test more SNPs than for others and then, the chances for spurious association are larger too. We performed the SNP significance analysis separately for time points 1 and 2 and then combined the \( P \)-values from the two time points using the Fisher method for those SNPs influencing protein abundance in the same direction. In this way, we got a single \( P \)-value per each protein. Finally, we call significant pQTLs based on a 10\% FDR using the R package \( q \)-value (Storey & Tibshirani, 2003).

**Overlap with eQTLs**

To check if the effect of our pQTLs on protein level was due to a change in gene expression, we looked for associations of the pQTL SNPs with gene expression measured using RNAseq technology in four tissues (fat, LCLs, skin and blood) in about 800 female twins (Buil et al., 2015). We analyzed each tissue and each gene separately. For each gene, we used a linear model to estimate the association between the pQTL and a normalized expression of each exon. We kept the best \( P \)-value per gene and estimated its statistical significance by comparison with a null distribution obtained by permuting the gene expression labels 10,000 times.

**Other bioinformatic analyses**

Hierarchical clustering analysis (HCA) was performed by Cluster 3.0 on the log-transformed, 2-dimensional-centered and normalized peptide intensities and visualized by Treeview. R software was used for plotting histograms. All the \( P \)-values indicating the significance of the data distribution difference were also reported by R using the Wilcoxon rank-sum test with continuity correction (the command is wilcox.test). The paired Wilcoxon test was used to compare the contribution of heritability and family component between two time points. The proteins significantly affected by the four biological processes was done using David bioinformatics resource (Huang da et al., 2009), where the enrichment analysis was performed by taking all the proteins in our human plasma spectral library as background.

The HDL protein list was extracted based on consensus summary of published HDL studies (Shah et al., 2013), whereas the non-HDL protein list contains those proteins that have not been supported as HDL protein in either of the studies (Shah et al., 2013).

**Data availability**

All the raw data of mass spectrometry measurements (SWATH-MS and shotgun), together with the input spectral library for human plasma proteome and OpenSWATH results can be freely downloaded from ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino et al., 2014) with the dataset identifier PXD001064. SNP genotyping data can be accessed with the permission of the TREC committee.

**Supplementary information** for this article is available online: http://msb.embopress.org

**References**


**Author contributions**

YL performed all the mass spectrometry experiments, analyzed the mass spectrometry data and wrote the paper. AB developed the linear mixed model under heavy discussion with YL and performed the quality control analysis and pQTL analysis. BC and LG involved in SWATH-MS data acquisition. LB and OV provide helpful inputs on the development of linear mixed model. GL collected the clinical information of the twins. RA, ED, YL and TS designed the study. RA supervised the study and wrote the paper.

**Conflict of interest**

The authors declare that they have no conflict of interest.


pipeline for high-throughput label-free quantitative proteomics. J Proteome Res 12: 1628–1644

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