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Progress Identifying and Analyzing the Human Proteome: 2021 Metrics from the HUPO Human Proteome Project

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Abstract

The 2021 Metrics of the HUPO Human Proteome Project (HPP) show that protein expression has now been credibly detected (neXtProt PE1 level) for 18,357 (92.8%) of the 19,778 predicted proteins coded in the human genome, a gain of 483 since 2020 from reports throughout the world reanalyzed by the HPP. Conversely, the number of neXtProt PE2, PE3, and PE4 missing proteins has been reduced by 478 to 1421. This represents remarkable progress on the proteome parts list. The utilization of proteomics in a broad array of biological and clinical studies likewise

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continues to expand with many important findings and effective integration with other omics platforms. We present highlights from the Immunopeptidomics, Glycoproteomics, Infectious Disease, Cardiovascular, Musculo-Skeletal, Liver, and Cancers B/D-HPP teams and from the Knowledgebase, Mass Spectrometry, Antibody Profiling, and Pathology resource pillars, as well as ethical considerations important to the clinical utilization of proteomics and protein biomarkers.

Graphical Abstract
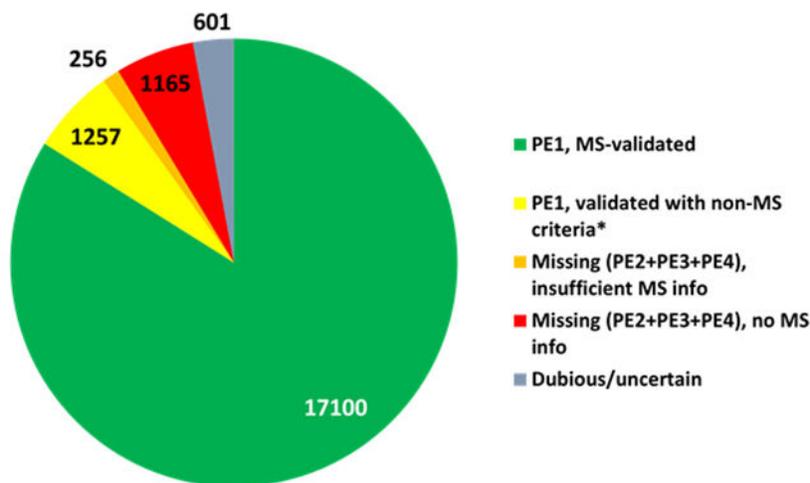
Keywords

Human Proteome Project (HPP); neXtProt protein existence (PE) metrics; missing proteins (MP); non-MS PE1 proteins; uncharacterized protein existence 1 (uPE1); Chromosome-centric HPP (C-HPP); Biology and Disease-HPP (B/D-HPP); PeptideAtlas; Mass Spectrometry Interactive Virtual Environment (MassIVE); Human Protein Atlas

PROGRESS ON THE HUMAN PROTEOME PARTS LIST

For more than a decade, the Human Proteome Project (HPP), the flagship initiative of the global Human Proteome Organization (HUPO), has pursued twin goals: credibly identifying the protein parts list, essentially, but not entirely, by mass spectrometry, and making proteomics an integral part of multi-omics studies of health and disease[1, 2]. The HPP consortium has stimulated international collaboration, data sharing, standardized reanalysis of datasets, guidelines for quality assurance, and progress in building and utilizing proteomics knowledge globally. The HPP is organized into 25 teams by chromosome and mitochondria, 19 teams by biological processes and diseases, and 4 resource pillars for antibody-based protein localization, mass spectrometry, knowledgebases, and pathology[3].

The chromosome-centric protein parts list is curated and documented by neXtProt[4]. Table 1 shows the annual progress from 13,975 proteins with protein-level evidence (PE1) as of 2012-02 to 18,357 PE1 proteins in the neXtProt release of 2021-02, including an increase of 473 in the past year from 2020 to 2021. This PE1 total now represents 92.8% of the PE1,2,3,4 predicted proteins from protein-coding genes. This is quite remarkable

because we have wondered for several years whether we might be approaching the limit of detection of several classes of very low abundance or membrane-embedded proteins. Moreover, we have applied progressively more stringent Guidelines for Interpretation of Mass Spectrometry Data[5, 6]. Conversely, Table 1 documents the reduction of PE2,3,4 "missing proteins" (classified based on expression of transcripts, expression in other species, or gene models, respectively, while lacking sufficient protein-level evidence) from 5511 in 2012-02 to 1421 in 2021-02, which is 7.2% of the PE1,2,3,4 total of 19,778.

Figure 1 presents a schematic flow diagram of the changes in the neXtProt PE levels for predicted proteins during the past year, from 2020-02 to 2021-02. neXtProt includes a PE5 category for dubious or uncertain genes, mostly pseudogenes, which we exclude from the HPP metrics. Of the 17,874 PE1 proteins in 2020, 17 were deleted due to changes in UniProtKB/Swiss-Prot that carried into neXtProt, especially merging entries; 19 were demoted to PE2,3,4 MPs and 4 to PE5; and 17,834 were carried forward to the 2021-02 release. Meanwhile, 61 new protein entries appeared in neXtProt, of which 40 were PE1, 5 PE2, 4 PE3, 0 PE4, and also 12 PE5; there are 18 immunoglobulin chains, 13 T Cell Receptors, and 30 miscellaneous proteins. Of the 61, 21 were based on non-MS evidence; the entire list appears as Supplementary Table 1.

From the point of view of the Missing Proteins, there were 1899 in the 2020-02 release (Table 1, Figure 1). Of those, 481 were elevated to PE1, a remarkably large number, including 331 from PE2, 106 from PE3, and 41 from PE4. Most of this very large gain comes from the inclusion of PPI data in Swiss-Prot or neXtProt. Swiss-Prot curates, validates, and integrates PPI data from publications and from IntAct for upgrading to PE1; that decision is then incorporated by neXtProt. In 2020 an IntAct PPI dataset drove the number of PPI-based PE1 entries in Swiss-Prot from 334 to 615. The pharmaceutical industry has increased its focus on PPI targets; PPI are entering into mainstream drug discovery. neXtProt incorporated a human PPI dataset mapped to the level of interacting domains manually annotated from the literature by ENYO Pharma SA, which resulted in 150 additional PE1 upgrades. As with all other data in neXtProt, these PPI data are "FAIRified": Findable using the neXtProt semantic SPARQL query tools; Accessible under CC-BY 4.0 license; Interoperable with all the data in neXtProt and many other resources using the same semantic web standards; and Reusable, since all publications and their experimental methods are documented using the PSI Molecular Interations (PSI-MI) controlled vocabulary.

For MS-based PE1 decisions, neXtProt incorporates peptide identifications from PeptideAtlas[7, 8] (see Table 1) and MassIVE and applies HPP guidelines for protein validation (at least two uniquely-mapping, non-nested peptides of 9 or more amino acids from a single source)[3, 5, 6].

UniProtKB now has 176 entries for functional immunoglobulin (Ig) genes in 3 clusters located on Chr 14 (50V, 23D, 6J, 9C = 88), Chr 2 (40V, 5J, 1C = 46), and Chr 22 (32V, 5J, and 5C = 42). The huge antibody repertoire of each human arises during B cell development though combinatorial V-(D)-J rearrangements, junctional diversity, and somatic hypermutation to yield about 10^{12} different immunoglobulins. UniProtKB relies

on IGMT (the international ImMunoGeneTics organization at CNRS/Montpellier, France) for immunoglobulin and T cell receptor nomenclature and data. Joint with IMGT-Gene-DB, UniProtKB/Swiss-Prot presents a representative set of full-length germline Ig protein sequences, with 15 entries showing the sequences of all C regions and 122 representing all V regions. Sequences for D (5aa) and J (15-30 aa) are extremely small. An Ig is encoded by 7 genes: IGHV, IGHD, IGHJ, IGHC for the H chain and IGKV, IGKJ, or IGKC for a kappa or KGLV, IGLJ or IGLC for a lambda chain. As for other human proteins, the sequences shown match translation of the reference genome (Genome Reference Consortium GRCh38/hg38). To the best of our knowledge, there is no plan to capture immunoglobulin heterogeneity in UniProtKB/Swiss-Prot; this is the mission of the specialized, comprehensive IMGT repertoire. Reciprocal links to IMGT from UniProtKB ensure easy navigation between resources (http://www.imgt.org/IMGTrepertoire/).

Of the 18,357 PE1 proteins, 17,100 (92.6%) are now based on mass spectrometry and 1257 (7.4%) are validated with non-MS protein evidence (Figure 2). Of the 17,100 MS-based PE1 proteins, 16,920 from the published datasets were reanalyzed and submitted to neXtProt by PeptideAtlas; 16,252 were reanalyzed by MassIVE and submitted to neXtProt; 16, 074 were in both PA and MassIVE; 17,097 in PA or MassIVE; 177 only in MassIVE and 846 only in PeptideAtlas; while 3 non-PE1 proteins meeting the HPP Guidelines 3.0 were added by neXtProt from PTM papers not reviewed by PA or MassIVE. Currently, 78% of neXtProt entries have at least one PTM associated, which represents a total of 191,837 PTM sites. Approximately half of the sites are phosphorylation sites from PeptideAtlas; the others were imported from UniProtKB or GlyConnect or manually annotated from publications (16,759 ubiquitin-like conjugation sites, 3,647 glycosylation sites, 3,354 phosphorylation sites, 2,468 acetylation sites and 1,174 methylation sites). Many more PTMs certainly are present in human samples, but their detection remains a challenge. The various resources named above, including neXtProt, make a constant effort to model and curate new PTM data in order to better capture the complexity of the human proteome.

Of the 1257 non-MS-based PE1 proteins, 62 are primarily based on Edman degradation, 30 on 3D structures in Protein Data Bank, 765 on protein-protein interactions, 42 on antibody studies, 133 on PTMs and processing, 6 on genetic mutations, and 219 from biochemical studies. Many are supported by multiple types of studies; those with MS data do not meet the HPP Guidelines.

As the Figure 2 pie chart and Figure 3 bar chart summarize, besides the 17,100 MS-based PE1 and 1257 non-MS-based PE1 proteins, there are now just 1421 PE2,3,4 missing proteins (in gray) that remain to be confidently detected; of these 256 have some MS data but insufficient to meet the Guidelines. The 2020 Metrics paper included a discussion of the features of the most challenging remaining missing proteins[2]; these challenges remain cogent.

A multi-year longitudinal depiction of the pie chart from the baseline in 2016 after application of the HPP Guidelines for Interpretation of MS Data v2.1 (and v3.0 in 2019) is presented in Figure 3. The detailed figures (related to Table 1) are provided for the 2016 base year and show the increment from 2020 to 2021.

Finally, Figure 4 shows the contribution of the top 9 new datasets in PeptideAtlas that generated the increment of 47 canonical proteins (Table 1). Three of these datasets come from the NCI CPTAC3 studies of colorectal cancers, HPV-negative head & neck squamous cell cancers, and glioblastomas.

A paper published too late to make the 2021 update of PeptideAtlas addressed the under-studied skin proteome and reported 10,701 proteins detected (though not using HPP Guidelines 3.0)[22]. This dataset will be subjected to analysis in the TransProteomicPipeline, with the HPP Guidelines, and be incorporated into the 2022 update of PeptideAtlas and neXtProt.

HIGHLIGHTS OF THE CHROMOSOME-CENTRIC HPP (C-HPP)

The C-HPP teams contributed 15 papers across a wide variety of topics and a lead editorial[22] to the JPR 2020 HPP 8th Special Issue (https://pubs.acs.org/toc/jprobs/19/12) following 20 published in the JPR 2019 HPP Special Issue. In addition, 61 papers were published elsewhere during 2019/2020. Progress on the MP50 missing proteins challenge, based on the work of the entire community, is documented in Tables 1 and 2.

Several chromosome teams (e.g., Chr 5 and Chr 12) are active in the Cancer Moonshot and CPTAC projects and successfully analyzing these data for MPs. Chromosome 6 initiated a directed search for PE1 proteins lacking MS evidence (non-MS PE1 proteins), with several identified by MS in human bone that met the HPP Guidelines for PE1 identification by MS[15]. A precision medicine molecular corrector drug was developed by Chr 6 team members that restored functional levels of a mutant protein isoform of MALT1[23]. Untreated, this mutant protein led to a rare immunodeficiency disease. The disease was phenotyped in a previous paper by proteomics and TAILS that led to this discovery and then the drug candidate.

Chromosome 10 has assembled one of the world's largest collections of full-length Gateway plasmids representing 90% of all human protein-coding genes and are distributing the collection through their repository and distribution web portal DNASU (dnasu.org). Chr 10 with Chr 5, 15, 16, and 19 teams have been providing the IVTT-compatible plasmids for missing proteins for IVTT-assisted SRM for many years and continue to generate more plasmids for the whole community. Currently, Chr 10 has full-length plasmids for 175 missing proteins.

A total of 15 C-HPP teams have now met the MP50 Challenge (Table 2). For examples, the number of PE2,3,4 missing proteins coded on Chr 17 has been reduced from 148 in 2016 to 58 in 2021, with reduction on Chr X from 145 to 51, corresponding to 90 and 94 new PE1 proteins, respectively. The CP50 Challenge to each C-HPP team[24] to characterize the function(s) of currently unannotated PE1 proteins has made modest progress, as the number of uPE1 proteins (1273 in 2021 *versus* 1254 in 2020) has not risen proportionately with the increase in total PE1 proteins. Table 2 shows the number of functionally unannotated PE1 proteins for each chromosome. In order to speed up work on the CP50 challenge, neXtProt has designed specific community pages to host manually verified protein function

predictions. Duek et al conducted a Functionathon with students at the University of Geneva and proposed functional annotation for seven interesting uPE1 proteins. This work complements the annotations using I-TASSER/CO-FACTOR[25]. The HPP teams and the broad community are encouraged to discuss the available predictions, to submit new ones, and to start planning validation experiments.

HIGHLIGHTS FROM THE B/D-HPP

The Biology and Disease branch of the Human Proteome Project (B/D-HPP) aims to characterize the biological functions of human proteins and to uncover drivers of disease development and outcomes of preventive and therapeutic interventions. The B/D-HPP integrates 19 international teams of scientists that focus on distinct areas of biological and clinical relevance, including immunity, infectious diseases, cancers, glycoproteomics, cardiovascular, skeletal muscle, and liver diseases, and nutrition (https://hupo.org/B/D-HPP). These research areas are expanded and updated depending on need and the significance of emerging topics. The teams implement proteomics within multidisciplinary biological and clinical studies and disseminate appropriate proteomics methods to the broader scientific community.

Immunopeptidomics.

The comprehensive characterization of Major Histocompatibility Complex (MHC)-associated peptides has experienced dramatic progress in recent years. Immunopeptidomics is expected to impact the treatment of cancers, infectious diseases, and autoimmunity. During the COVID-19 pandemic, the SARS-CoV-2 peptidome and human leukocyte antigen (HLA) peptides associated with T cell responses were reported[26-28], supporting the design of vaccines. Proteogenomics/bioinformatics analyses of HLA-bound peptides detected immunogenic cancer-specific targets from non-coding genes, transposable elements, novel ORFs, and bacterial sources, which could be targeted by cancer immunotherapy[29-33]. For example, 16S rRNA gene sequencing and HLA peptidomics demonstrated the presentation of bacterial peptides by tumor cells from melanoma metastases; intracellular bacteria may impact immune responses[29]. Integration of exome sequencing, single-cell transcriptomics, ribosome profiling, and MS analyses identified tumor-specific non-canonical HLA peptides[30]. Other studies are exploiting data-independent acquisition (DIA), MS/MS deep learning, and automation[34-39], and large-scale resources are becoming available[18, 40].

Glycoproteomics.

HLA-bound peptides identified on dendritic cells upon exposure to SARS-CoV-2 spike (S) protein displayed trimmed glycan residues[27]. Overall, the standardization of proteomics workflows, which is needed for reproducible, traceable, and transferable measurements, remains an important focus for the glycoproteomics team. The initiative MIRAGE (Minimum Information Required for a Glycomics Experiment) has been designing guidelines that improve the reporting and reproducibility of glycoanalytical methods, such as for liquid chromatography[41]. Standardization has been enhanced through a recent interlaboratory study targeting monoclonal antibodies for biopharmaceuticals development[42]. This collaborative strategy revealed a significant

increase of paucimannosidic glycans in several types of cancers, including liver, colorectal, prostate, chronic lymphocytic leukemia, and glioblastoma cells[43]. Moreover, the platform Glyconnect (https://glyconnect.expasy.org/) allows gathering, monitoring, integration, and visualization of data to facilitate interpretation of collected glycoscience data[44]. neXtProt is integrating glycosylation sites from Glyconnect. The critical nature of protein glycosylation in human health and disease progression is illustrated by the discovery of fibronectin as a target of the glycosyltransferase fukutin-related protein (FKRP) and its link with muscular dystrophy[45], and the role of hyper-truncated Asn355- and Asn391-glycans in myeloperoxidase activity and neutrophil-mediated immunity[45].

Post-Translational Modifications (PTMs) in Infectious Diseases.

The characterization of PTMs as molecular toggles with critical roles in processes required for cellular homeostasis or linked to disease development has been a shared interest of B/D-HPP teams, including glycoproteomics, cancers, and cardiovascular and infectious diseases. ID investigators discovered lamin B1 site-specific acetylation at the nuclear periphery as a regulatory hub functioning in host defense during viral infection and in choice of DNA damage pathways during cell cycle progression[46]. An MS-based approach defined phosphoribosylated peptides alongside phosphopeptides on distinct proteoforms in lipopolysaccharide (LPS)-stimulated macrophages, pointing to possible functional cross-talk between PTMs[47]. Thermal proximity coaggregation (TPCA) and thermal proteome profiling have characterized overall impacts, both pro- and anti-viral, on protein-protein interactions of human cytomegalovirus[48]. SARS-CoV-2[49], and herpes simplex virus type 1[50]. The TPCA method captured dynamic enzyme-substrate interactions, leading to discovery of a functional link between the viral DNA interferon inducible protein 16 (IFI16) and the DNA-dependent protein kinase (DNA-PK)[50], enhanced computationally[51]. Many studies examined the biology, pathogenesis, and therapeutic intervention opportunities for SARS-CoV-2 infections, including immunohistochemistry on autopsy lung and proteomics on blood from COVID-19 subjects with acute respiratory distress syndrome (ARDS)[52,55] and various bacterial infections[53-56].

Cardiovascular Disorders.

Brisk progress has been made by Cardiovascular B/D-HPP researchers in both technology development and biological investigations. The Van Eyk group at Cedars-Sinai has reported a refined protocol and instructions for automated programmable plasma protein digestion in 96-well plate format, which improved the accuracy, precision, and reproducibility of targeted and discovery proteomics assays for clinical implementation[57]. The Kirk group at Loyola University used ubiquitination proteomics to reveal a new link between the BAG3 protein and myofilament turnover in the setting of heart failure[58]. The Ge group at University of Wisconsin made advances with top-down proteomics to characterize proteoform permutations and discover that different genetic variants associated with hypertrophic cardiomyopathy funnel into common patterns of altered sarcomeric proteoforms that are predictive of disease phenotypes and may represent common intervention targets[59]. The Gundry group at the University of Nebraska Medical Center pioneered a new reference glycan structure library for the human heart and demonstrated that specific changes in glycan structure motifs underpin cardiomyocyte differentiation[60] and probably other

critical cardiac functions. The Mayr group at King's College London used targeted mass spectrometry and other techniques to show that human PCSK9 is primarily associated with HDL in the circulation, which will facilitate ongoing development of therapeutics targeting PCSK9 to lower serum LDL-cholesterol[60].

Musculo-Skeletal.

This B/D-HPP team has focused on understanding the molecular mechanisms that underlie the decline of skeletal muscle strength with aging, which is a primary cause of mobility loss and frailty in the elderly. Such mechanisms have been investigated using transcriptomics[61], ribosome profiling[62], proteomics in muscle biopsies[63], and plasma proteomics[64]. These multiomic studies revealed that the older human muscle is characterized by deranged energy metabolism, a pro-inflammatory environment, increased proteolysis, and changes in alternative splicing.

Liver.

A major area of interest within the Human Liver Proteome Project team has been the study of proximal biofluids as a valuable information source to assess disease setting, status, and progression. A specific procedure to analyze the human bile proteome[65] aimed to discriminate biliary stenosis cases resulting from cholangiocarcinoma or from benign conditions; the combination of proteomics and metabolomics resulted in a panel of 5 proteins and 10 lipids that discriminated between patients with and without these cancers with unprecedented accuracy[66]. Combined analyses also elucidated pathogenic mechanisms underlying liver disorders. Methionine adenosyltransferase 1A (MAT1A) and the product of its activity, S-adenosylmethionine (SAMe), are keys to preserving a well differentiated and quiescent liver. MAT1A has been recognized as a tumor suppressor in hepatocellular carcinomas, mediated at least in part by interaction with FOXM1[67]. Moreover, Kilanczyk et al have shown that SAMe promotes antioxidant and glutathionylation processes in cholangiocytes that prevent the autoimmune response in patients with primary biliary cholangitis[68]. Finally, several tools extract and visualize protein-centric functional networks, such as PINE[69], and infer biological functions of uncharacterized proteins, uPE, as defined by the C-HPP, based on gene expression correlation and the PageRank algorithm UPEFinder[70].

Cancers.

Cancer B/D-HPP investigators are working within large collaborative teams as part of the U.S. National Cancer Institute Clinical Proteomic Tumor Analysis Consortium (CPTAC) and the International Cancer Proteomics Consortium (ICPC). From these large-scale collaborative efforts, proteo(geno)mes are being characterized to shed light on different aspects of cancer biology, immunology, and pathology and to propose precisely targeted therapeutic interventions, as shown during this past year for glioblastomas[21], head and neck cancers[20], pediatric brain cancers[71], breast cancers[72], serous ovarian carcinomas[73], and lung adenocarcinomas[74], as reviewed by Nice[75] from the HPP Pathology Pillar. To aid the analysis of large-scale quantitative proteomic datasets, these studies have been accompanied by continued improvement of web servers for data evaluation, visualization, and modeling (*e.g.,* ProteomeExpert and BatchServer)[76, 77].

Lastly, to facilitate the dissemination of results and newly-developed methodologies during the pandemic-imposed virtual setting this past year, the B/D-HPP has inaugurated webinars to bring together scientists from different B/D-HPP teams, open to the public. The first was dedicated to "PTMs in Human Disease" (https://www.hupo.org/Webinars-and-Virtual-Presentations).

HIGHLIGHTS FROM THE KNOWLEDGEBASE AND MASS SPECTROMETRY RESOURCE PILLARS

The Human Plasma Proteome and the Human PhosphoProteome

An updated report on the Human Plasma Proteome led by Schwenk and Deutsch (under review for this Special Issue) provides a comprehensive update on the status of the human blood plasma proteome, including several special topics—extracellular vesicles, COVID-19 studies, coagulation, proteogenomics, and ageing. The Human Plasma PeptideAtlas Build of 2021-01 now comprises 4389 canonical human proteins, and the Human Blood Extracellular Vesicle PeptideAtlas 2021-07 has 2757 canonical proteins detected from extracellular vesicles circulating in plasma, of which 2047 are in common and 703 detected only in the EV datasets. These resource pillars are also developing a Human PhosphoPeptideAtlas for phosphosites on serine, threonine, and tyrosine residues of proteins that can be identified and be related to the phosphorylating kinase, the de-phosphorylating phosphatase, cross-talk with other post-translational modifications, and activation of downstream pathways. Many of the precision oncology medications target these proteins and the dynamic phosphoproteome. Kalyuzhnyy et al[78] profiled the phosphoproteome to estimate the true extent of protein phosphorylation.

Hoopmann et al[79] reported the results of the Phosphopeptide Challenge organized by the HPP Mass Spectrometry Resource Pillar. A standard set of 94 phosphopeptides with phosphorylation at Ser, Thr, or Tyr residues, and their non-phosphorylated counterparts, mixed at different ratios in a neat sample and in a yeast tryptic digest background, was prepared and provided to about 100 laboratories, of which 22 reported data back. The peptides were synthesized and contributed to the HPP by SynPeptide of Shanghai, China, and IMAC beads were provided by ReSyn Biosciences of Johannesburg, South Africa. Each lab analyzed both samples with their own choice of fractionation and purification methods, instruments, and software; they reported identification and site localization of the peptides, relative abundances, and enrichment in the yeast background. The MS Pillar team then reanalyzed all of the submitted data with a single software pipeline (Trans-Proteomics Pipeline) and discussed the challenges and successes in correct identification and localization of phosphosites. True positive, false positive, and false negative identifications from each lab were analyzed across the many variables. Given the salience of phosphopeptide analyses in proteogenomic analyses, like those of CPTAC (above), these results will be quite useful. The data were uploaded to MassIVE with ProteomeXchange identifier PXD020801.

HIGHLIGHTS FROM THE PATHOLOGY RESOURCE PILLAR

The Pathology Pillar, co-led by Michael Roehrl and Edouard Nice, has been active in organizing many meetings and reaching out to other components of the HPP to stimulate future collaborations. Publications from the past year from the Roehrl Laboratory at Memorial Sloan Kettering Cancer Center include DEAD-box RNA helicase protein DDX21 as a prognosis marker for early stage colorectal cancers[80], autoantigen profiles of A549 lung and Hep-2 cells[81, 82], MS-based absolute quantification of amyloid proteins[83], and proteome-based pathology for precision medicine[84]; see also Nice[75].

Complementary publications from the Aebersold Laboratory and colleagues at ETH-Zurich show important insights from proteogenomic analyses. Mehnert et al[85] determined the effects of specific mutations in the gene for the Dual-Specificity Tyrosine Phosphorylation-regulated Kinase Dyrk2 on various levels of the proteome, altering the composition, topology, and activity of the kinase complex and the phosphoproteomic state of the cell through altered protein-protein interactions. There is extensive plasticity of the molecular responses to gene mutations. The Tumor Profiling (TuPro) Study workflow[86] examines how complex mechanisms evolve in tumors as biological responses involve the tumor microenvironment, cellular heterogeneity, and cell-cell interactions within a tumor mass. These molecular pathology studies were performed on specimens from patients with metastatic melanomas, metastatic epithelial ovarian cancers, and acute myeloid leukemias, with two million single cells per patient profiled across six technologies, together with bulk approaches. Digital pathology and imaging mass cytometry provide quantitative, single-cell, spatially resolved data particularly useful for predicting the success of therapies like immune checkpoint inhibitors that depend upon direct cell-cell interactions.

HIGHLIGHTS FROM THE ANTIBODY PILLAR/HUMAN PROTEIN ATLAS

The Antibody Resource Pillar is tightly linked to the Human Protein Atlas (HPA) project[87], a major open-access knowledge resource focusing on spatial proteomics, antibody-based technologies, and integration with transcriptomics. In 2020, the HPA celebrated 20 years since the start of the project, and released a dedicated section and booklet summarizing all the major scientific milestones (www.proteinatlas.org/about/history). HPA v20 also includes a new main section, where the HPA takes the leap into single cell transcriptomics in the Single Cell Type Atlas. This new section constitutes an important complement to the Tissue Atlas, allowing integration of mRNA and protein levels in single cell types. Another recent major milestone is the release of a Cell Cycle Atlas[88]. By utilizing the novel data in the Single Cell Type Atlas, the cell type-specific expression patterns observed with the antibodies can be confirmed, taking antibody validation to the next level and opening up more detailed studies focusing on particular cell types[89].

In addition to the large-scale effort mapping all the human proteins in various organs, tissues, cells and organelles, a major emphasis of the Antibody Resource Pillar and the HPA is developing stringent criteria for antibody validation. These criteria were recently applied in a thorough study analyzing the body-wide expression profile of the SARS-CoV-2 receptor ACE2[90], where both previously described and novel cell type-specific expression

patterns could be confirmed with high confidence, as highlighted in the 2020 HPP Metrics paper[2]. Surprisingly, the expression level of ACE2 in human airway epithelia was shown to be low, which is in contrast to previous studies and highlights the importance of proper criteria for validation in antibody-based studies. Based on a workflow from the HPA project, a laboratory-based antibody assay for detection of SARS-CoV-2 specific IgG in serum and plasma has been set up, showing 99.7% sensitivity and 100% specificity[91] and also useful detection in saliva[92].

Meanwhile, Sivertsson et al[93] explored antibody validation from a proteome-wide perspective, comparing level of confidence of the antibody-based data in the HPA with protein evidence levels. As expected, a relatively high proportion of the neXtProt missing proteins (PE2-PE4) lacked available antibodies or were only targeted by antibodies of lower reliability, suggesting that this group of proteins that remain to be detected is challenging for many reasons. Nevertheless, a subset of 56 missing proteins, as well as 171 proteins of unknown function, that were suggested, based on transcriptomics levels, to be higher expressed in specific tissues and especially male tissues/testis, were targeted by antibodies of high quality. This group of missing proteins for which reliable antibodies are available constitutes an important starting point for further assessment of evidence of existence, as well as for focused studies combining mass spectrometry with antibody-based proteomics for orthogonal validation of the specific protein identifications.

Beyond the production of antibodies for immunohistochemical and immunofluorescent characterization of expression of the human proteome, there has been substantial development of ELISA-type assays based on antibodies[94] or aptamers[95] as affinity binders to detect arrays of proteins in human fluids to complement or compete with targeted or non-targeted mass spectrometry-based detection and quantification. For example, Magis et al[96] used 13 panels of Olink assays in a longitudinal scientific wellness study from which several cancer biomarkers were shown to identify individuals who would later be diagnosed with metastatic cancers. It is important to obtain results from analyses with each of the platforms on the same samples, both for orthogonal quality control and for better understanding of the sensitivity, specificity, advantages, and limitations of each for plasma and for selected tissue specimens. In an important step forward to align the standards and metrics of new protein assay systems, users of both Olink and Somascan participated in the 2021 meeting of the HUPO Proteomics Standards Initiative[97, 98] to move toward developing guidelines for quality of detection surrounding these affinity based assays, following the model of the MS Data Interpretation Guidelines v2.1[5] and v3.0[5, 6].

Yet another development in the utilization of antibodies is the programming of kinetically controlled biomolecular probes to design antibodies directed at "undruggable" targets, starting with ion channels (specifically, transient receptor potential vanilloid 1, TRPV1) and KRAS mutations through which apoptosis can be induced[99]. Very limited protease action is a key step in this protocol.

ETHICAL CONSIDERATIONS FROM PROTEOME-BASED BIOMARKER DEVELOPMENTS

With the remarkable progress in proteomic technologies and throughput and aims to develop protein biomarkers for diagnosis, prognosis, and choice of therapies, authors of a pair of articles from the Matthias Mann Laboratory seek to stimulate the proteomics and multi-omics communities to think deeply about the ethical considerations that arise from research and then clinical use of protein biomarkers from mass spectrometry-based or affinity-based proteomes. Geyer et al[100] utilized the data set from a 12-month study of 42 individuals in a weight loss protocol[101] to show that the longitudinal pattern of individual protein concentrations and sequences of variant peptide alleles could identify each individual. Porsdam Mann et al[102] illustrated applications of the four core principles of bioethics (in parentheses) as follows: informed consent to elicit data-sharing preferences (autonomy); protection of the privacy of personal health information (justice); withholding of non-actionable incidental findings (non-maleficence); and report and explanation of actionable incidental findings (beneficence). They performed a systematic review of the literature on "ethics and clinical proteomics" and identified 10 normative themes, including standards and quality control, integration of new laboratory and computer technologies, conflicting rights and duties, incidental findings, and international legal and regulatory protections and requirements. The 1975 Asilomar Conference on the development of recombinant DNA research and the current international consensus to limit uses of CRISPR-Cas9 gene editing to somatic cells in humans are notable examples of deep engagement of the biomedical community with the larger public.

Meanwhile, Nobis et al[103] addressed bioethics in the context of cancer biomarker research with a focus on actual and potential benefits and harms, respect for participants, and fair, just, and moral research practices. Specifically, they emphasized the validation of biomarkers, confidentiality, identifiability of specimens, data protection, and the return of research results. The HUPO HPP is committed to share, analyze, and curate data from the various HPP projects ethically and responsibly.

OUTLOOK FOR THE NEXT SEVERAL YEARS

The extraordinary advances described above and in the papers celebrating the 10th anniversary of the HPP[1-3] suggest that the HPP is asymptotically approaching its main stated goal, a high stringency blueprint of the human proteome based on high quality data and transparent, error-controlled computational methods for peptide and protein inference. It therefore seems an opportune time for the HPP to initiate community discussion of major challenges facing proteomics and to develop plans for the HPP to address them over the next years.

To this end, the HPP Scientific Advisory Board (chaired now by Ruedi Aebersold) organized a session on the occasion of the HPP Day on 22 October 2020 in the closing session of the virtual HUPO Annual Congress. The discussions centered around the following challenges that represent exciting opportunities for proteomics:

1. <u>Complexity of the proteome</u>. The HPP effort has helped the community achieve confident identification of one (sometimes several) specific translation products for most protein-coding genes, corresponding to the 92.8% classified as PE1 proteins. In reality, the number of protein species that constitute the proteome – proteoforms - is massively expanded compared to the number of protein-coding genes by mechanisms including alternative splicing, protein processing, and post-translational modifications. The proteoforms universe is largely unexplored. Furthermore, proteins assume different conformations in 3D and associate with other biomolecules to form macromolecular complexes that often constitute functional units. Importantly, these latter properties of the proteome cannot be presently predicted from their polypeptide sequence. Recently a range of methods has become available to measure them. The confident measurement of these properties of the proteome therefore will provide an exclusive opportunity for proteomics to discover new biology not revealed by DNA and RNA studies.

2. <u>The Translation of Molecular Maps</u>. The core proteome already mapped and the forthcoming maps of proteoforms and complexes need to be translated into biological function and phenotypes. It is generally well accepted that proteins either by themselves or in the form of modules are responsible for most biological functions. Knowing the proteins as chemical entities does not fully explain their biological functions. The determination of the biological function of the proteoforms and macromolecular complexes that constitute the proteome is a major opportunity for the proteomics community. In that regard and to focus the task at hand, it is useful to consider different levels of function, including the biochemical and cell biological functions of proteins. The biochemical, catalytic function of a protein, exemplified by the phosphotransferase activity of a protein kinase or the proteolytic activity of a protease, is of fundamental importance for the translation of molecular measurement into an understanding of molecular processes and mechanisms. The cell biological functions of proteins reflect their roles in the context of the living cell. The cellular function of proteins needs to explain, *e.g.,* the set of proteins that are phosphorylated by a specific kinase— and dephosphorylated by a specific phosphatase – in a particular cellular state and then the impact of these events on the biochemical processes of the cell and then higher levels of organization. The exploration of the cellular functions of proteins and protein modules presents an outstanding opportunity for proteomics with large implications for basic and translational research.

3. <u>Mainstreaming Proteomics in Life Sciences Research</u>. Increased dissemination and uptake of proteomics techniques and knowledge resources as a mainstream component of life science research has been a goal for many years. It has been apparent for some time that proteomics uptake has been slow compared to, *e.g.,* genomic or imaging-based approaches, even though it is now widely acknowledged that proteomic data are particularly and uniquely informative. Addressing the bottlenecks that prevent more rapid uptake and dissemination of proteomics presents a high yield opportunity for proteomics and developers of

technologies and sample processing methods for a much broader community of users in an era of precision medicine.

The 2020 workshop discussions provided a rich source of ideas and information to further develop a HPP roadmap for the next few years. The three anniversary articles in *Nature Communications*[1], *Journal of Proteome Research*[2], and *Molecular & Cellular Proteomics*[3] each painted a vision of strategic developments for the coming decade. Over the last few months, the HPP Executive Committee has continued these discussions and is presently planning community workshops with the goal of arriving by 2022 at a consensus HPP roadmap and actionable research plan for the next several years.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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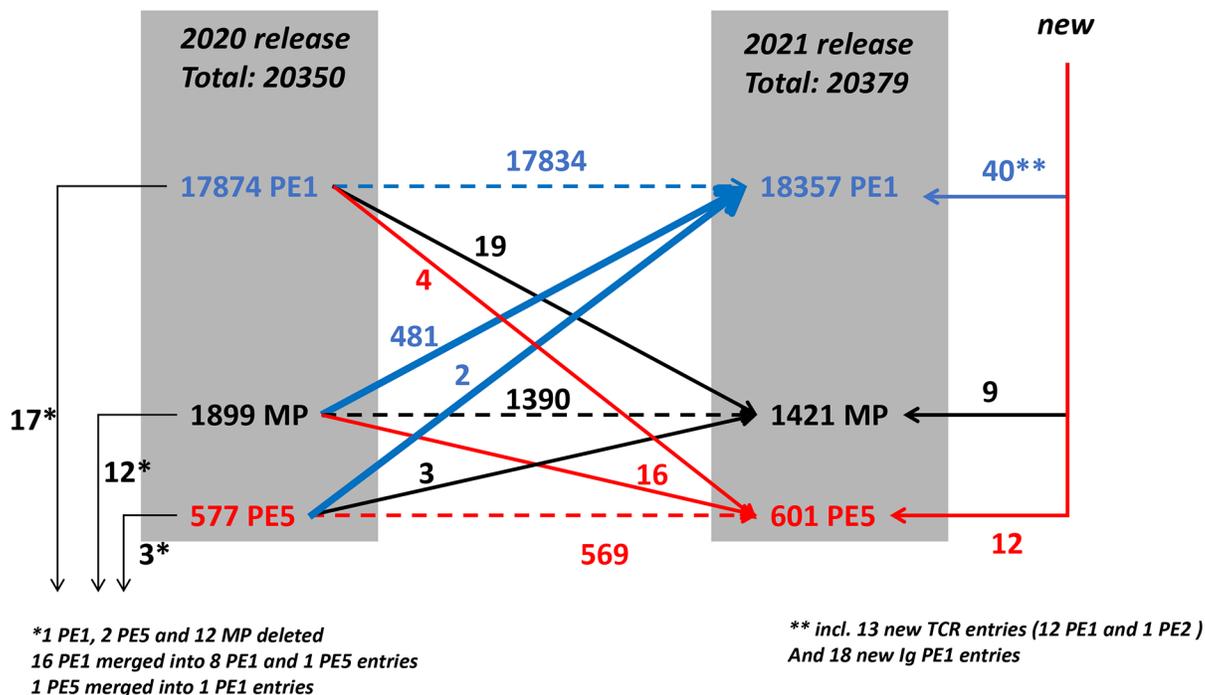
Figure 1.
Flow diagram of changes in numbers of PE 1,2,3,4,5 classes of predicted proteins from 2020-02 to 2021-02 neXtProt release. Blue arrows indicate increases to PE1 (blue); red arrows indicate demotions from PE1 or PE2,3,4 MPs to PE5 (red); black arrows show increases to MPs (black). In the margins are shown new entries to and removals from neXtProt (see text).
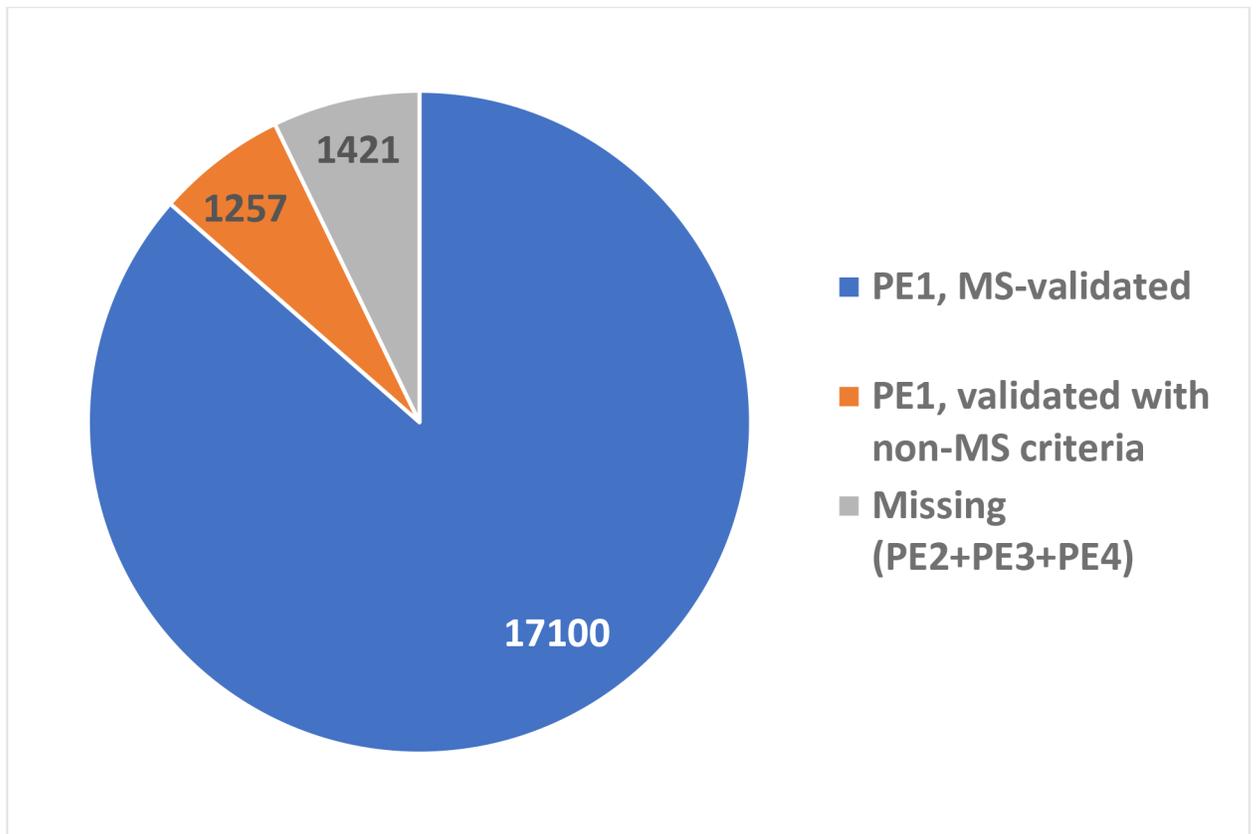
Figure 2.
Pie chart of MS-based PE1 (blue), non-MS-based PE1 (orange), and PE2,3,4 predicted proteins lacking any or sufficient protein-level evidence, shown as PE2 as of neXtProt release 2021-02. The progress in these metrics since 2016 is depicted in detail in Figure 3.
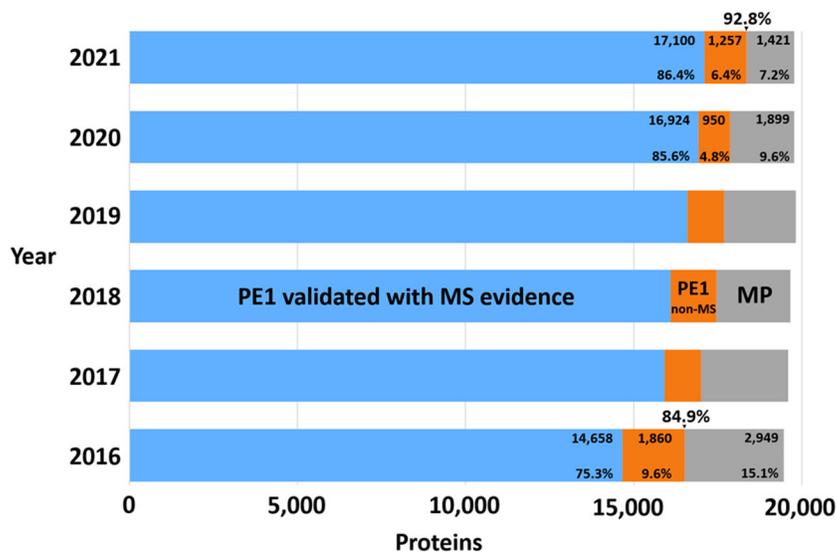
Figure 3.
Bar chart showing the growth in numbers of PE1 proteins based on MS evidence (blue), the initial diminution in numbers of PE1 proteins based on non-MS data (as many of these proteins were identified with MS) with a large increase for 2021 from PPI datasets (orange) (see discussion of Figure 1), and the striking, progressive reduction in PE2,3,4 missing proteins (grey). The numbers 84.9% and 92.8% above 2016 and 2021 represent the percentage of all PE 1,2,3,4 proteins that are PE1.
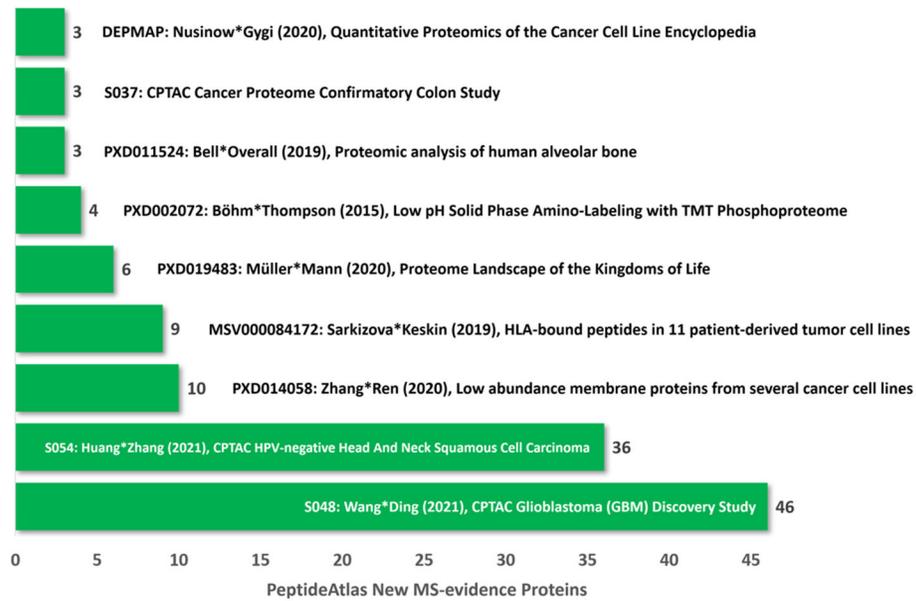
Figure 4.
Nine datasets registered in ProteomeXchange[9] and incorporated into PeptideAtlas after reanalysis with the Trans-Proteomic Pipeline[10-12] in the 2021-02 build that contributed the most new PeptideAtlas canonical proteins[13-21]. The newly MS-based PE1 proteins arose as new PE1 proteins that were previously PE2,3,4 (gray in Figure 3) or from previously non-MS-based PE1 (orange in Figure 3). Each dataset is labeled with its identifier in the respective database, the first and last authors' names, publication year, number of new canonical proteins, and major biological features. As of 5/17/21, there are 6330 human proteomic datasets in ProteomeXchange.

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Table 1.

neXtProt protein existence evidence levels from 2012 to 2021 showing progress in identifying PE1 proteins and PeptideAtlas canonical proteins. More stringent guidelines were imposed in 2016.

PE Level	2012-02	2013-09	2014-10	2016-01	2017-01	2018-01	2019-01	2020-01	2021-02
1: Evidence at protein level	13,975	15,646	16,491	16,518	17,008	17,470	17,694	17,874	**18,357**
2: Evidence at transcript level	5205	3570	2647	2290	1939	1660	1548	1596	**1265**
3: Inferred from homology	218	187	214	565	563	452	510	253	**147**
4: Predicted	88	87	87	94	77	74	71	50	**9**
MP = PE2 + PE3 + PE4	5511	3844	2948	2949	2579	2186	2129	1899	**1421**
Human PeptideAtlas canonical proteins	12,509	13,377	14,928	14,569	15,173	15,798	16,293	16,655	**16,702**

[a]PE1/PE1+2+3+4 = 18,357/19,778 = 92.8%

[b]PE 2+3+4 = 1421 "missing proteins" as of 2021-02.

Table 2.

Chromosome-by-Chromosome Status of Predicted Proteins in neXtProt 2021-02-18

Chr	PE1	PE2	PE3	PE4	PE2+3+4	PE1/PE1-4	% PE1/PE1-4	Net Decrease PE2+3+4 (2016 to 2021)	uPE1 Proteins
1	1859	139	21	2	162	1859/2021	92.00%	147 (309/162)	148
2	1228	45	3	1	49	1228/1277	96.20%	85 (134/49)	80
3	1005	52	5	0	57	1005/1062	94.50%	84 (141/57)	60
4	701	35	11	0	46	701/747	93.80%	49 (95/46)	49
5	835	26	2	0	28	835/863	96.80%	94 (122/28)	55
6	940	54	4	1	59	940/999	92.80%	77 (136/59)	60
7	863	105	6	1	112	863/975	89.70%	25 (137/112)	52
8	625	34	5	0	39	625/664	94.30%	56 (95/39)	39
9	706	58	4	0	62	706/768	91.20%	67 (129/62)	67
10	681	47	0	1	48	681/729	92.80%	67 (115/48)	50
11	1069	169	53	0	222	1069/1291	82.70%	97 (319/222)	73
12	959	52	2	0	54	959/1013	94.90%	65 (119/54)	45
13	311	10	1	0	11	311/322	96.60%	32 (43/11)	29
14	632	75	12	2	89	632/721	88.80%	4 (93/89)	37
15	535	40	0	0	40	535/575	93.40%	33 (73/40)	34
16	773	44	0	1	45	773/818	94.50%	54 (99/45)	51
17	1087	55	3	0	58	1087/1145	94.70%	90 (148/58)	64
18	255	9	0	0	9	255/264	96.20%	15 (24/9)	11
19	1318	79	8	0	87	1318/1405	93.90%	174 (261/87)	79
20	504	31	0	0	0	504/535	93.70%	51 (82/31)	40
21	204	27	2	0	0	204/233	87.60%	20 (49/29)	11
22	455	23	5	0	28	455/483	95.80%	36 (64/28)	33
X	764	51	0	0	51	764/815	93.60%	94 (145/51)	105
Y	37	3	0	0	3	37/40	92.50%	13 (16/3)	1
Mito	15	0	0	0	0	15/15	100.00%	0 (0/0)	
Unk	2	2	0	0	2	4-Feb	50.00%	0 (2/2)	
ALL	**18357**	**1265**	**147**	**9**	**1421**	**18357/19778**	**92.80%**	**1528 (2949/1421)**	**1273**
Sums	18363	1271	147	9					

NOTE: There are discrepancies between the true total numbers of proteins in each PE category (**ALL**) and the Sums because six proteins are derived from two genes on two different chromosomes, and thus appear twice under the per-chromosome table values.