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Quantitative mass spectrometry-based proteomics in the era of model-informed drug development: Applications in translational pharmacology and recommendations for best practice

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ABSTRACT

Quantitative translation of the fate and action of a drug in the body is facilitated by models that allow extrapolation of *in vitro* measurements (such as the rate of metabolism, active transport across membranes, inhibition of enzymes and receptor occupancy) to *in vivo* consequences (intensity and duration of drug effects). These models use various physiological parameters, including data that describe the expression levels of pharmacologically relevant enzymes, transporters and receptors in tissues and *in vitro* systems. Immunoquantification approaches have traditionally been used to determine protein expression levels, generally providing relative quantification data with compromised selectivity and reproducibility. More recently, the development of several quantitative proteomic techniques, fuelled by advances in state-of-the-art mass spectrometry, has led to generating a wealth of qualitative and quantitative data. These data are currently used for various quantitative systems pharmacology applications, with the ultimate goal of conducting virtual clinical trials to inform clinical studies, especially when assessments are difficult to conduct on patients. In this review, we explore available quantitative proteomic methods, discuss their main applications in translational pharmacology and offer recommendations for selecting and implementing proteomic techniques.

Contents

Abbreviations: AQUA, absolute quantification standards; CYP, cytochrome P450; DDA, data-dependent acquisition; DDI, drug-drug interaction; DIA, data-independent acquisition; iTRAQ, isobaric tags for relative and absolute quantitation; IVIVE, *in vitro-in vivo* extrapolation; LC, liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; MRM, multiple reaction monitoring; MS, mass spectrometry; MS^E, mass spectrometry by collision energy alternation; *m/z*, mass-to-charge ratio; PBPK, physiology-based pharmacokinetics; PD, pharmacodynamics; PK, pharmacokinetics; PRM, parallel reaction monitoring; PSAQ, protein standards for absolute quantification; PTM, post-translational modification; Q, quadrupole; QC, quality control; QconCAT, quantitative concatemers; QSP, quantitative systems pharmacology; SILAC, stable isotope labeling in cell culture; SNP, single nucleotide polymorphism; SRM, selected reaction monitoring; SWATH, sequential window acquisition of all theoretical fragment mass spectra; TMT, tandem mass tags; TOF, time-of-flight; TPA, total protein approach; UGT, uridine 5′-diphospho-glucuronosyltransferase.

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1. Introduction

Translational pharmacology requires extrapolation of *in vitro* observations to predict the outcome of therapy *in vivo* using various scaling factors measured in tissues and relevant *in vitro* systems (Rostami-Hodjegan, 2012). When extrapolating measurements made *in vitro* (e.g. Km, Vmax, Jmax), functional data may be used as scalars when selective probes are available, for example in the case of several cytochrome P450 (CYP) (Walsky & Obach, 2004) and uridine 5′-diphospho-glucuronosyltransferase (UGT) enzymes (Achour et al., 2017; Walsky et al., 2012). However, owing to a lack of specific substrates for many enzymes and for the majority of transporters and receptors, the use of abundance data remains the preferred approach for *in vitro-in vivo* extrapolation (IVIVE), facilitated by analytical methods that can quantify the levels of individual proteins in heterogeneous biological matrices. Over the past two decades, quantitative proteomics based on liquid chromatography in conjunction with mass spectrometry (LC-MS) has replaced traditional immunoquantitative methods, such as Western blotting and enzyme-linked immunosorbent assays (ELISA) (Aebersold, Burlingame, & Bradshaw, 2013), mainly because traditional techniques require purified protein standards and specific antibodies for each target, which are not always available.

Pharmacologically active enzymes and transporters tend to have high sequence homology and most of these proteins are found at very low amounts within the membranes of tissues and cellular systems (Vildhede, Wiśniewski, Norén, Karlgren, & Artursson, 2015). Highly selective and sensitive mass spectrometry techniques are therefore ideal for implementation in pharmacology applications (Al Feteisi, Achour, Rostami-hodjegan, & Barber, 2015; Heikkinen, Lignet, Cutler, & Parrott, 2015). LC-MS analysis offers various other advantages including reproducibility, high throughput and the ability to multiplex measurements. This allows simultaneous detection and quantification of dilute amounts of a large number of proteins (hundreds to thousands) in complex biological systems (Ong & Mann, 2005). Quantitative proteomic techniques have therefore been implemented by different laboratories worldwide for various pharmacology applications, leading to improved extrapolation of drug pharmacokinetics (Doki et al., 2018; Kumar et al., 2018) and better understanding of the effects various factors, including age (Ladumor et al., 2019; van Groen et al., 2018), ethnicity (Kawakami et al., 2011; Ladumor et al., 2019; Peng, Bacon, Zheng, Guo, & Wang, 2015), and genetics (Bhatt et al., 2019; Peng et al., 2015; Weiß et al., 2018) on the expression of enzymes and transporters.

The typical aim of a proteomic experiment is to characterize the entire set of proteins expressed in a particular system (global proteomics) or to target a specified set of proteins for quantification (targeted proteomics) (Gillet, Leitner, & Aebersold, 2016). These two types of proteomic analysis require specific considerations for robust analysis to be achieved (Prasad et al., 2019). In this review, we explore state-of-the-art mass spectrometry-based proteomic methods, both global and targeted, used for the characterization of drug metabolizing and transporting proteins as well as drug targets, and discuss their advantages, limitations, caveats for implementation and their main applications in translational pharmacokinetics (PK) and pharmacodynamics (PD).

2. Overview of a typical quantitative proteomic experiment

The quantitative proteomic workflow can be customized for the type of biological sample and the target proteins to be quantified; however, routinely applied bottom-up methods tend to follow generally similar steps (Fig. 1A). A biological sample (tissues, cell lines or biofluids) is processed by cell lysis or homogenization, often followed by enrichment of specific fractions (e.g. microsomes, cytosol, S9, plasma membrane, mitochondrial fraction) (Fig. 1B) prior to protein solubilization and digestion (Drozdzik et al., 2014; Harwood, Russell, Neuhoff, Warhurst, & Rostami-Hodjegan, 2014; Wiśniewski, Wegler, & Artursson, 2016). The variable array of available samples requires consideration of the effects of the type of sample and subsequent processing on end-point protein abundance measurement (Bhatt & Prasad, 2018).

Whole cell lysates or tissue homogenates can be used for the quantification of various pharmacologically relevant proteins (Wegler et al., 2017). When enriched systems are required, the localization of the target protein is critical to the decision of which fraction to use (Wiśniewski, Wegler, & Artursson, 2016). Cytosolic proteins (e.g. alcohol/aldehyde dehydrogenases, sulfotransferases) are best quantified in cytosol or S9 fractions (Prasad et al., 2018). Membrane-bound reticular proteins (e.g. CYPs and UGTs) are enriched in microsomal membrane fractions (Chen, Zane, Thakker, & Wang, 2016). Enzymes localized in the reticular lumen (e.g. carboxylesterases) can be quantified in microsomes; however, a proportion of these proteins is expected to be lost into the cytosol during sample processing and therefore these proteins are quantified more accurately in S9 fractions (consisting of microsomes and cytosol) provided the target proteins are sufficiently abundant (Prasad et al., 2018; Wang, Shi, & Zhu, 2019). Transporters and PD-relevant targets, such as receptors, protein phosphatases and kinases, can be found in the plasma membrane (Batth et al., 2018; Ohtsuki et al., 2012), and therefore cell membrane-enriched fractions can be used for these applications. Detailed sub-cellular location information can be found in various databases, including Gene Ontology (www.geneontology.org) and UniProt (www.uniprot.org).

Bottom-up proteomic techniques rely on quantitative analysis of unique (proteotypic) peptides used as surrogates for target proteins (Gillet et al., 2016). Sample proteins are digested using specific proteases, typically trypsin or lysyl endopeptidase (LysC), independently or in combination (Achour & Barber, 2013; Wiśniewski & Mann, 2012). Other proteases, such as chymotrypsin, can be used for specific applications, such as increased depth and reproducibility of analysis (Wiśniewski, Wegler, & Artursson, 2019). Sample digestion can be done in gel, in solution or using filter-aided sample preparation (FASP) (Fallon et al., 2008; Langenfeld, Zanger, Jung, Meyer, & Marcus, 2009; Wiśniewski, Zougman, Nagaraj, & Mann, 2009). Complementary data are expected to be generated when several protein preparation workflows are used (Al Feteisi et al., 2018; Choksawangkarn, Edwards, Wang, Gutierrez, & Fenselau, 2012). After digestion, peptides are desalted, enriched, separated by liquid chromatography (LC) and analyzed using mass spectrometry (MS). Additional separation prior to mass spectrometry can be performed using ion mobility (Achour, Al Feteisi, Lanucara, Rostami-Hodjegan, & Barber, 2017; Distler et al., 2014). Multiple quantitative MS and data acquisition approaches can be used (Fig 1C), depending on the aim of the experiment and the availability of instrumentation (Smith, Martins-de-Souza, & Fioramonte, 2019). Targeted and global methodologies are routinely used to identify and quantify expression levels of pharmacologically-relevant proteins. Standards are added at different stages of the proteomic workflow (Fig 1A). Data acquisition is followed by data analysis and interpretation, often facilitated by vendor or open-source software. Assessment of the performance of various software packages used for targeted and global proteomics was previously reported (Cox & Mann, 2008; Röst et al., 2014; Tommi, Suomi, & Elo, 2018)

Several quality control (QC) steps are required at certain stages of the experiment. Assessment of the quality of sample processing during homogenization and fractionation is required to ensure maximum recovery of protein, normally using colorimetric/fluorometric protein assays. Assessment of the digestion efficiency is done before LC-MS analysis; this is achieved by evaluating time-dependent release of peptides in targeted experiments or by monitoring the rate of missed cleavage in global experiments. Finally, the reliability of the proteomic quantification technique depends on the performance of the LC-MS system, which can be assessed using internal standards and well-characterized QC samples (Bhatt & Prasad, 2018; Prasad et al., 2019).

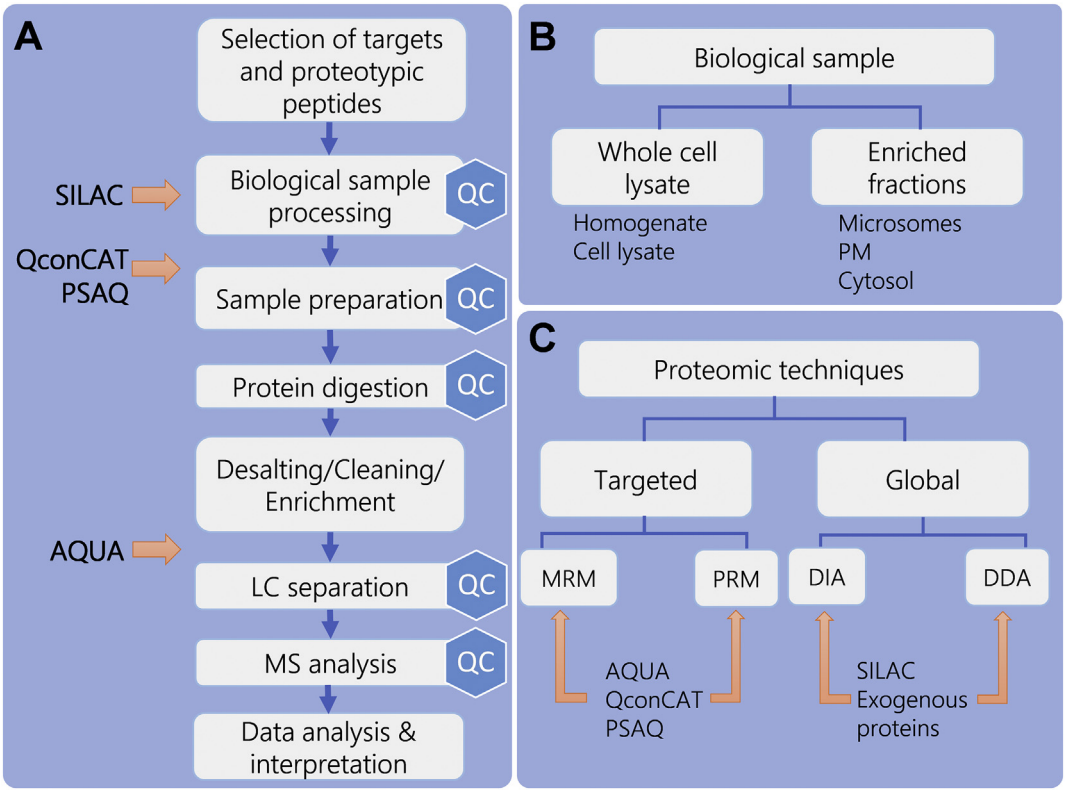

Fig. 1. Overview of the experimental quantitative proteomic workflow. A. Basic proteomic strategy starting from selection of targets and sample preparation, followed by LC-MS analysis, and finally data analysis/interpretation. Protein digestion relies on proteases, such as trypsin and lysyl endopeptidase (LysC), and can be done in solution, in gel or using filter-aided sample preparation (FASP). Standards are added at different stages of sample preparation. SILAC mixtures represent isotopically labeled proteomes; QconCAT and PSAQ protein standards are added to samples prior to protein digestion; AQUA peptide standards are added before LC-MS analysis. Several quality control (QC) steps are required throughout the workflow. B. The two main types of samples used to generate proteomic data, whole cell lysates (cell and tissue homogenates) and enriched fractions (e.g. microsomes, plasma membrane, cytosol, mitochondrial fractions or S9 fractions). C. The main types of proteomic techniques (targeted and global) and data acquisition methods (MRM/PRM for targeted proteomics and DDA/DIA for global proteomics). Red arrows show the steps where standards are introduced. Abbreviations: AQUA, absolute quantification peptide standards; DDA, data-dependent acquisition; DIA, data-independent acquisition; MRM; multiple reaction monitoring; QC, quality control; QconCAT, quantitative concatemers; PM, plasma membrane; PRM, parallel reaction monitoring; PSAQ, protein standards for absolute quantification; SILAC, stable isotope labeling by amino acids in cell culture.

3. Targeted quantitative proteomic methods

Targeted methods are in many ways superior to global methods for the quantification of specific proteins of interest that are known to be expressed in a particular system. The use of targeted proteomics with enriched fractions (e.g. plasma membrane, microsomes) offers highly reproducible measurements of proteins expressed at low levels. The workflow of quantification using these methods starts with identifying the target proteins of interest, followed by selection of proteotypic peptides used as surrogates to quantify the selected targets. These methods require stable isotope labeled (SIL) internal standards for absolute quantification. Generally, MS platforms used for targeted techniques include triple quadrupole (QqQ), quadrupole/time-of-flight (Q-TOF) and hybrid Orbitrap mass spectrometers. Table 1 summarizes the advantages and limitations of targeted proteomic methods. The types of targeted acquisition methods are discussed below.

3.1. Selected/multiple reaction monitoring (SRM/MRM)

Selected or multiple reaction monitoring (SRM/MRM) is the most commonly used targeted proteomic method in biological and pharmacological research (Gillette & Carr, 2013; Kitteringham, Jenkins, Lane, Elliott, & Park, 2009). In MRM, a peptide and a selected set of its fragment ions (transitions) are monitored by mass filtering on a triple quadrupole instrument (Carr et al., 2014). The technique is routinely used with internal SIL standards, and heavy (standard) and light (analyte) ions are analyzed simultaneously. This technique offers several advantages including multiplexed analysis, high throughput, reproducibility, sensitivity and wide dynamic range (Aebersold et al., 2013; Carr et al., 2014). The sensitivity achieved by this method makes it ideal when samples are small, e.g. biopsies (Vrana, Whittington, Nautiyal, & Prasad, 2017). The limitations of targeted techniques include the requirement for extensive method development and the selection of suitable targets and surrogate peptides. Low abundance analyte proteins are not accurately quantified and interference can occur due to the use of pre-defined mass filters and low resolution mass analyzers (Gillette & Carr, 2013; Kitteringham et al., 2009).

Several applications of this technique have been reported including determination of inter-individual variability in drug-metabolizing enzymes and transporters (Gröer et al., 2014; Kumar et al., 2015; Margaillan et al., 2015), prediction of variability in clearance (Vildhede et al., 2016) and drug-drug interactions (DDIs) (Doki et al., 2018), determination of inter-species differences in transporter expression at the blood-brain barrier (Al Feteisi et al., 2018; Hoshi et al., 2013), characterization of various hepatocyte-based *in vitro* systems (Kumar et al., 2019; Schaefer et al., 2012), region-specific transporter expression in the brain (Billington et al., 2019), kidney (Prasad et al., 2016) and intestine (Drozdzik et al., 2014), region-specific enzyme expression in the kidney (Knights et al., 2016), quantification of biomarkers in biological fluids, such as plasma and urine (Abbatiello et al., 2015) and assessment of the effects of disease on different organs (Al-Majdoub et al., 2019; Billington et al., 2018; Prasad et al., 2018).

Table 1
The overall aims, advantages and limitations of various proteomic data acquisition methods: targeted (MRM, PRM), global data-dependent acquisition (DDA) and data-independent acquisition (DIA) techniques.

Method	Advantages	Disadvantages
Targeted techniques (MRM, PRM) Aim: Robust quantification of a selected set of proteins, known to be expressed in a particular system	• High sensitivity and reproducibility • Simple data analysis • Allows relative and absolute quantification; SIL standards address matrix effects • High resolution instruments are not required for MRM • High selectivity with PRM	• Limited resolution and selectivity with MRM • Limited number of target proteins (10-50 targets per single analysis) • Requirement of prior knowledge of target proteins • Requirement for synthesis of internal standards • Targeted methods cannot be used for discovery of novel targets or pathways
Global data-dependent acquisition (DDA) techniques Aim: discovery proteomics and proteome-wide quantification	• Simple method setup • High proteome coverage • Internal SIL standards are not needed • Allows relative and absolute quantification (with spiked standards or TPA approach) • PTMs can be characterized using global data • Data can provide guidance for targeted quantification	• Bias toward highly expressed proteins and compromised reproducibility for low abundance proteins • Sensitive to changes in LC-MS conditions due to requirement for long runs • Absolute quantification is less reliable than targeted methods • Requirement of instrument with high-end specifications • Selectivity and sensitivity are compromised
Global data-independent acquisition (DIA) techniques Aim: discovery proteomics and proteome-wide quantification. In the case of sequential window methods (SWATH), the aim can also be set to the quantification of a limited number of target proteins	• Moderate/high precision of peptide quantification. • Wide breadth of peptide identification and quantification leading to high target coverage (typically higher than DDA) • Amenable to discovery and quantitative applications • Provides rich data for targeted methods, including peptide information, fragment information, PTMs and potentially SNPs	• Complex and convoluted data • SWATH requires multiple steps to compile spectral libraries, with many parameters to optimize • Requirement of instrument with high-end specifications • Requirement for specialist software and high computational power for analysis

MRM, multiple reaction monitoring; PRM, parallel reaction monitoring; PTM, post-translational modifications; SIL, stable isotope labeled; SNP, single-nucleotide polymorphism; SWATH, sequential window acquisition of all theoretical fragment mass spectra; TPA, total protein approach

3.2. Parallel reaction monitoring (PRM)

Parallel reaction monitoring (PRM) is a recently introduced targeted method with higher specificity than MRM (Gallien, Duriez, Demeure, & Domon, 2013; Schiffmann et al., 2014) because of the use of high-end mass spectrometers, such as Orbitrap (Gallien et al., 2012; Peterson, Russell, Bailey, Westphall, & Coon, 2012) and quadrupole/time-of-flight (Schilling et al., 2015) platforms, offering high resolution and high mass accuracy. The principle of PRM is based on simultaneous monitoring of all (precursor ion/fragment ion) transitions of a targeted peptide arising from both standard and sample, in parallel at the MS and tandem MS (MS/MS) levels. By contrast, the MRM approach monitors only pre-defined fragments. The combination of full scan mode, high resolution and high mass accuracy makes PRM a very attractive method, especially for the analysis of complex biological matrices. PRM requires less time for method development and is less prone to interference than MRM owing to the availability of a higher number of quantifiable fragments (Gallien, Bourmaud, Kim, & Domon, 2014; Ronsein et al., 2015). Because of the large number of monitored transitions, the sensitivity of PRM is sometimes reduced relative to MRM, and the requirement of high resolving power makes the technique less widely applicable (Gallien et al., 2014). Comparable performance by MRM and PRM has recently been demonstrated (Nakamura et al., 2016; Ronsein et al., 2015). Reported applications of PRM-MS include plasma biomarker analysis (Kim et al., 2015), quantification of enzyme variants (Shi et al., 2018), and characterization of PK-relevant proteins in liver, kidney and intestine pools (Nakamura et al., 2016).

3.3. Accurate mass and retention time (AMRT)

Quantification (relative or absolute) based on accurate mass and retention time (AMRT) is a simple and rapid method (Silva et al., 2005). This method is less widely used than MRM and PRM techniques and relies on measurement of precursor ion intensity of analyte and standard peptides at a predefined mass (m/z ratio) and retention time. Confirmation of the peptides identities is carried out after fragmentation at the MS/MS level. This method can be used in conjunction with global proteomic methods to quantify selected targets in proteome-wide analyses. Because AMRT relies on the parent ion intensity in the MS scan, its efficiency is dependent on reproducible peptide separation (by LC) and the use of high resolution mass analyzers (MS). In addition, only a limited number of moderate to high abundance proteins can be quantified because of limited sensitivity. This technique was applied to measuring protein abundance in human serum (Silva et al., 2005) and assessment of disease perturbations in the expression of transporters at the blood-brain barrier (Al-Majdoub et al., 2019).

4. Standards for targeted proteomics

Absolute quantification is typically achieved by targeted techniques that use SIL peptides or proteins as standards or calibrants (Calderón-Celis, Encinar, & Sanz-Medel, 2018). These standards represent heavy versions of the surrogate peptides selected to quantify the target proteins. Standards are synthesized chemically or biologically and incorporate a heavy isotope label (^{13}C, ^{15}N), which allows distinction between analyte (light) and standard (heavy) by mass spectrometry. The types of standards routinely used in targeted quantitative proteomics include absolute quantification (AQUA) peptides, quantitative concatemers (QconCAT) and protein standards for absolute quantification (PSAQ). A summary of the characteristics of these standards is shown in Table 2.

The selection of standard peptide sequences is a critical step and follows previously reported criteria (Kamiie et al., 2008). These criteria can also be applied to select surrogate peptides in global proteomic methods (Prasad et al., 2019). Generally accepted requirements include:

- Proteotypic sequence: unique to the protein of interest with distinct mass (m/z) and fragmentation pattern (MS/MS); isobaric and isomeric sequences are avoided.
- Cleavable by proteases used in quantitative proteomics: the sequence should not be mapped to transmembrane domains; absence of closely occurring cleavage sites in the target protein sequence (e.g. arginine (R) and lysine (K) in the case of trypsin).
- Detectable by LC-MS: optimal hydrophobicity (LC) and ionizability (MS); absence of known single nucleotide polymorphism (SNP) and post-translational modification (PTM); optimal length (7-25 amino acids depending on the MS platform).
- Stable: not susceptible to chemical modification during storage and handling, including oxidation of methionine (M) and deamidation of asparagine/glutamine (N/Q).

Table 2
Characteristics of standards used in targeted proteomic methods (AQUA, QconCAT and PSAQ) and their analytical performance

	AQUA	QconCAT	PSAQ
Description	Chemically synthesized isotope labeled peptides	Biologically synthesized sequence of concatenated isotope labeled peptides	Intact isotope labeled recombinant protein
Commercial availability	Available	Available	Available
Digestion evaluation	Necessary	Necessary	Not Necessary but desirable
Number of target proteins	One for each standard	Up to 50 per standard protein	One for each standard
Cost	Low, depending on the number of targets	Moderate	High
Considerations for synthesis	Subject to stability issues during chemical synthesis	Subject to failure of expression	Subject to failure of expression
Addition in the experimental workflow	Post-digestion	Before solubilization and digestion	Before solubilization and digestion
Compatible proteomic techniques	MRM PRM	MRM PRM AMRT	MRM PRM
Performance of targeted methods	Highly reproducible Multiplexed	Highly reproducible Multiplexed Ideal for stoichiometric analysis	Highly reproducible Accurate
SNP and stoichiometric analysis	Possible; requires QC	Yes	No
Analysis of PTMs	Yes	No	No

AMRT, accurate mass and retention time mass spectrometry; MRM, multiple reaction monitoring; PRM, parallel reaction monitoring; PTM, post-translational modifications; QC, quality control; SNP, single-nucleotide polymorphism

These general selection criteria can be customized for different biological applications. For example, peptides with known PTMs and SNPs are targeted if the biological question requires such stoichiometric analysis. Allele-specific protein quantification was demonstrated recently for the assessment of significant genetic variations in CYP and UGT enzymes (Russell et al., 2013; Shi et al., 2018).

4.1. Absolute quantification (AQUA) peptide standards

SIL peptides or AQUA standards are chemically synthesized isotope labeled standard peptides with sequences specific to the target proteins. High quality and high purity peptides are available commercially in isotopically labeled form, making them easily accessible for large scale studies (Kettenbach, Rush, & Gerber, 2011; Kirkpatrick, Gerber, & Gygi, 2005). A known amount of the AQUA peptide is introduced into the sample at a late stage of sample preparation, usually after protein digestion. AQUA standards can be applied with MRM or PRM techniques, making these targeted techniques very useful when screening a specific protein in a large number of samples as a clinical test or when the quantification of a small set of proteins is desirable (Smith et al., 2019). AQUA can also be applied to the elucidation of PTMs, such as phosphorylation (Kettenbach et al., 2011). However, synthesis and quantification of standards for large scale studies is expensive and time-consuming (Al Feteisi, Achour, Barber, & Rostami-Hodjegan, 2015). The need to store peptides can be limiting as they tend to precipitate during long-term storage, which leads to inconsistent quantification (Mirzaei, McBee, Watts, & Aebersold, 2008). AQUA peptides are normally added to the sample directly before LC-MS analysis and the accuracy of quantification by the AQUA method can therefore be affected by analyte peptide loss during sample preparation (Havliš & Shevchenko, 2004). We recommend addition of standards to the samples before pre-fractionation and desalting so that equal loss of standard and analyte peptides is incurred from the mixture.

The AQUA-MRM approach is the most widely used quantification method in pharmacokinetic research and has been used to quantify various enzymes and transporters in different human tissues. Quantified enzymes include CYP and UGT enzymes in liver (Cieślak et al., 2016; Fallon, Neubert, Hyland, Goosen, & Smith, 2013; Hansen et al., 2019; Ohtsuki et al., 2012; Prasad et al., 2018; Sato et al., 2014; Sato, Nagata, Kawamura, Miyashita, & Usui, 2012; Weiß et al., 2018), intestine (Drozdzik et al., 2018; Gröer et al., 2014; Harbourt et al., 2012; Sato et al., 2014) and Kidney (Harbourt et al., 2012; Knights et al., 2016; Sato et al., 2014). In brain, the AQUA-MRM workflow was used to quantify CYPs, glutathione S-transferases (GSTs) and catechol O-methyltransferase (COMT) (Shawahna et al., 2011). Non-CYP and non-UGT drug-metabolizing enzymes quantified by this method include liver flavin-containing monooxygenases (FMOs), sulfotransferases (SULTs), aldehyde oxidase 1 and alcohol and aldehyde dehydrogenases (Bhatt, Gaedigk, Pearce, Leeder, & Prasad, 2017; Chen et al., 2016; Fu et al., 2013; Yoshitake, McKay-Daily, Tanaka, & Huang, 2017). In additions, drug transporters were successfully quantified using this quantitative strategy in various tissues, including liver (Prasad et al., 2013; Wegler et al., 2017), intestine (Drozdzik et al., 2014; Gröer et al., 2013), kidney (Prasad, Johnson, et al., 2016), brain (Billington et al., 2019; Shawahna et al., 2011; Uchida et al., 2011) and lung (Fallon, Houvig, Booth-Genthe, & Smith, 2018).

4.2. Quantitative concatemers (QconCAT)

QconCAT is a concatenated set of peptides expressed recombinantly from an artificial gene. The host organism is usually *E. coli* grown in culture media, supplemented with labeled amino acids, usually $^{13}C_6$-lysine and $^{13}C_6$-arginine. QconCATs are available commercially but can also be expressed in-house at relatively reasonable costs (Russell et al., 2013). The QconCAT protein is added to the sample at a known concentration (estimated using an unlabeled peptide corresponding to a standard peptide within the QconCAT) prior to digestion and can be used with several targeted techniques (MRM, PRM, AMRT). A single QconCAT can be designed to quantify several proteins (up to 50), making it amenable to multiplexing and higher coverage of protein targets. QconCAT ensures a strict 1:1 stoichiometry making it particularly advantageous for determining polymorphisms (Russell et al., 2013; Shi et al., 2018) and establishing protein-protein inter-correlations (Achour, Russell, Barber, & Rostami-Hodjegan, 2014). The development of QconCAT constructs is time-consuming and most worthwhile when a significant number of proteins (10–50) are to be quantified in a large number of samples. The QconCAT-MRM workflow has been successfully used to quantify hepatic drug-metabolizing enzymes (Achour, Russell, et al., 2014; Shi et al., 2018; Wang et al., 2015; Wang et al., 2019) as well as transporters in liver (Wegler et al., 2017), intestine (Harwood et al., 2015; Harwood et al., 2016a) and brain microvessels (Al Feteisi et al., 2018; Al-Majdoub et al., 2019).

Complete cleavage of peptides in the digestion process is, of course, essential, and there has been some interest in the use of 'flanking' sequences to make the environment of the peptides more analyte-like so that incomplete digestion will better resemble digestion efficiency of the target proteins (Cheung, Anderson, Wang, & Turko, 2015; Kito, Ota, Fujita, & Ito, 2007). Although this idea is attractive in theory, the

claim of comparable digestion efficiency between standard and analyte proteins is yet to be tested. We have preferred to optimize the digestion process so that there is complete release of peptides from the QconCAT and as far as possible of the target proteins (Achour et al., 2015; Al-Majdoub, Carroll, Gaskell, & Barber, 2014).

There is always the possibility of expression failure of a QconCAT, and this has been addressed in several ways (Achour et al., 2015; Russell et al., 2013). Experience indicates that smaller QconCATs are generally expressed more efficiently than larger constructs and ideally QconCATs should be below 100 kDa in size (Brownridge et al., 2011). The use of a small, insoluble tag, such as a ribosomal construct (Al-Majdoub et al., 2014) can force a QconCAT to express in insoluble form in inclusion bodies, from which it may be readily isolated (Russell et al., 2013). More radically, to address the issue of low yield and expression failure of larger QconCATs, multiplexed efficient expression of recombinant QconCATs (MEERCAT) was recently introduced to serve as standards for large scale protein quantification. The QconCATs are expressed in cell-free medium, with advantages such as expression efficiency, cost-effectiveness and ability to monitor the number of expressed QconCATs (Takemori et al., 2017).

4.3. Protein standards for absolute quantification (PSAQ)

A PSAQ standard is similar in concept to a QconCAT, but consists of an intact isotopically labeled recombinant protein added at a known concentration to the sample under investigation early in the sample preparation workflow. When a PSAQ standard is employed to quantify an unmodified protein, it can control for solubilization efficiency, digestion and LC-MS conditions; digestion discrepancies are avoided as PSAQ conserves the native context of the target peptides (Chen et al., 2017). This approach is particularly advantageous when quantifying low abundance, soluble targets in clinical samples (Adrait et al., 2012; Dupuis, Hennekinne, Garin, & Brun, 2008). However, PSAQ is only applicable to a small number of proteins; the development of such standards is prohibitively expensive and requires rigorous quality control (Al Feteisi, Achour, Barber, & Rostami-Hodjegan, 2015). This technique is not useful for assessing PTMs, identifying inter-correlations or multiplexed quantification of a large number of targets (Smith et al., 2019). The application of PSAQ in the quantification of drug-metabolizing enzymes and drug transporters in human tissue is yet to be reported. In biomarker research, this method was successfully used to quantify enzymes useful as indicators of cardiovascular disease (Huillet et al., 2012) and acute kidney injury (Gilquin et al., 2017) in biological fluids.

5. Global quantitative proteomic methods

Global untargeted proteomic approaches are routinely used for assessment of protein expression profiles, biomarker discovery, and identification and quantification of a large number of target proteins. Global approaches offer a wide dynamic range and broad proteome coverage while targeted approaches offer higher precision and accuracy. Proteome-wide quantification by global methods is routinely performed either by stable isotope labeling of sample proteins or peptides, e.g. stable isotope labeling by amino acids in cell culture (SILAC) and isobaric tags for relative and absolute quantitation (iTRAQ) (Ong et al., 2002; Wiese, Reidegeld, Meyer, & Warscheid, 2007), or by label-free analysis of the entire identifiable proteome (Silva, Gorenstein, Li, Vissers, & Geromanos, 2006; Vildhede et al., 2015).

In metabolic labeling methods, such as SILAC, samples are labeled with amino acids (e.g. arginine, lysine or leucine) carrying a stable isotope label (^{13}C, ^{15}N) and pooled before further sample processing, thus minimizing bias due to handling. The ratios of light to heavy peptide signals at defined retention times are used to relatively quantify protein expression differences between control and treatment conditions. Recent developments in labeling technology increased the ability of SILAC to multiplex from 2 samples (treatment versus control) to 6 samples (Merrill et al., 2014). SILAC is best suited for induction studies, elucidation of drug effects on protein expression (Kurokawa et al., 2019; Zhang, Xiao, & Wang, 2017), and analysis of post-translational modifications, such as relative quantification of phosphorylated proteins and identification of novel phosphorylation sites (Ibarrola, Kalume, Gronborg, Iwahori, & Pandey, 2003). In addition, SILAC has been used to prepare labeled standard mixtures for targeted proteomics (Geiger, Cox, Ostasiewicz, Wisniewski, & Mann, 2010). These labeled standards are added to analyte samples before protein digestion (Fig 1A), demonstrating similar performance to AQUA standards (Prasad & Unadkat, 2014). Metabolic labeling of whole animals, such as rodents, represents a recent extension of SILAC, with various applications in pharmacology research, such as the direct quantification of liver drug-metabolizing enzymes (MacLeod et al., 2015).

Chemical labeling methods, such as iTRAQ and tandem mass tags (TMT), are used at the peptide level after proteolytic digestion of sample proteins. Chemical tags react with amine groups and unique reporter ions are released upon fragmentation in MS/MS analysis (Ross et al., 2004). Unlike SILAC, chemical labeling can be used to analyze up to 8 samples and 11 samples in the same pool using iTRAQ and TMT reagents, respectively. Chemical labeling methods in conjunction with global proteomics demonstrated comparable performance to targeted AQUA-MRM methodology (Vildhede et al., 2018). Applications of chemical labeling include quantification of hepatic drug-metabolizing enzymes and drug transporters (Vildhede et al., 2018), characterization of plasma proteins in acute renal rejection (Freue et al., 2010), biomarker identification for breast cancer (Meiqun, Zifan, Kehuan, & Zhengzhi, 2011), eye disease (Linghu et al., 2017) and gum disease (Tsuchida et al., 2013), and relative quantification of proteins in Alzheimer's disease (Morales, Lachén-Montes, Ibáñez-Vea, Santamaría, & Fernández-Irigoyen, 2017). It is worth noting that proteome-wide labeling methods (SILAC/iTRAQ/TMT) are more aligned with applications that require relative quantification.

In label-free methods, normalization of measurements uses either unlabeled exogenous protein references or the total protein approach (TPA). Exogenous proteins include various protein standards distinct from the target proteome; for example, quantification of human enzymes can employ bovine serum albumin or yeast alcohol dehydrogenase at known concentrations (Silva et al., 2006). The TPA method uses the total intensity of peptide peaks belonging to a certain protein relative to the total intensity of all quantifiable peptides in the analyzed proteome (Wiśniewski et al., 2012). Both methods have previously been used to quantify human liver enzymes and transporters (Achour, Al Feteisi, et al., 2017; Couto et al., 2019; Vildhede et al., 2015).

Global proteomic techniques are generally carried out using Q-TOF or Orbitrap instruments. To correct for changes in MS conditions over long analyses, sophisticated correction and chromatographic alignment procedures are used to compensate for retention time shifts and to avoid mismatching peptide peaks across runs (Ludwig et al., 2018). Data acquisition methods used in global proteomics include data-dependent acquisition (DDA) and data-independent acquisition (DIA). DDA represents the standard shotgun approach widely used for whole-proteome analysis (Geromanos et al., 2009). On the other hand, the more recent DIA approach can generate more depth of analysis and broader proteome coverage, especially when window acquisition approaches, such as sequential window acquisition of all theoretical fragment mass spectra (SWATH), are used (Hu, Noble, & Wolf-Yadlin, 2016; Smith et al., 2019). A summary of the advantages and limitations of global proteomic methods is presented in Table 1.

5.1. Data-dependent acquisition (DDA)

In DDA, the initial scan of peptide peaks is used for the selection of peptides for fragmentation depending on their ion intensity, with the most abundant ions being selected preferentially. The main advantages

of DDA are its flexibility and broad proteome coverage compared to targeted methods. DDA proteomics can identify thousands of proteins and provide reliable relative quantification across samples (Hu et al., 2016). DDA can also be used for absolute quantification using suitable exogenous protein standards (Silva et al., 2006). However, this method is less precise in comparison with targeted quantitative methods as low abundance peptides are not detected reproducibly, leading to bias toward high abundance proteins (Hu et al., 2016; Michalski, Cox, & Mann, 2011; Wegler et al., 2017). The performance of this method declines as sample complexity increases (Bilbao et al., 2015; Geromanos et al., 2009).

Q-TOF or Orbitrap mass analyzers are normally used and data are interpreted using software packages, such as MaxQuant, Progenesis or Peaks. DDA data analysis can be performed either by spectral counting or by ion abundance/intensity (Ishihama et al., 2005; Silva et al., 2006), with ion intensity preferred owing to its higher accuracy and reproducibility (Distler et al., 2014; Prasad et al., 2019). Importantly, to ensure robust quantification, consistency in sample preparation and stability of LC-MS conditions are required. DDA shotgun methodology was successfully used for the quantification of transporters and receptors at the blood-brain barrier (Al-Majdoub et al., 2019) and for profiling various enzymes and transporters in liver tissue (Couto et al., 2019; Vildhede et al., 2018, 2015; Wegler et al., 2017; Wiśniewski et al., 2019) and hepatocyte-based *in vitro* systems (Vildhede et al., 2015; Wiśniewski, Vildhede, Norén, & Artursson, 2016).

5.2. Data-independent acquisition (DIA)

DIA was proposed to address the limitations of DDA in relation to limited depth of analysis and biased quantification. In DIA, all precursor ions within a selected mass range are fragmented and analyzed (Hu et al., 2016). Theoretically, this method identifies all detectable peptides within the selected mass range and is therefore less biased towards high abundance proteins. However, the generated data tend to be highly complex and specialized software is required for data deconvolution post-acquisition (Ludwig et al., 2018). DIA combines the advantage of broad proteome coverage offered by DDA methods and highly reproducible quantification, typically achieved by targeted techniques (Gillet et al., 2016; Hu et al., 2016). The most widely used DIA approaches include MS^E (Distler et al., 2014; Silva et al., 2006) and SWATH (Gillet et al., 2012). MS^E is a collision energy alternation method that uses a range of collision energies over a *m/z* window, leading to high- and low-energy fragmentation (Distler et al., 2014). The deconvoluted spectra are searched against a protein database for identification, while quantification can be done using an unlabeled standard protein. The applications of MS^E include relative and absolute label-free quantification of proteins (Bilbao et al., 2015). For example, this method was successfully used for quantitative profiling of various drug-metabolizing enzymes in human liver (Achour, Al Feteisi, et al., 2017).

In methods that use fragmentation windows, such as SWATH-MS, instead of fragmenting the entire set of precursor ions in a particular scan, small *m/z* windows can be selected for fragmentation and acquisition (Gillet et al., 2012). This potentially reduces the complexity of data and theoretically improves analytical depth and coverage. SWATH is widely applied using Q-TOF and Orbitrap mass analyzers, and data are processed by sophisticated pipelines, such as the open-source, cross-platform software OpenSWATH (Röst et al., 2014). The main advantages of SWATH are its compatibility with the analysis of low abundance subproteomes and PTMs, such as acetylation and glycosylation (Keller et al., 2016), and its high reproducibility and consistency owing to peptide-centric scoring analysis (Ludwig et al., 2018). SWATH is therefore particularly applicable when wide proteome coverage, high consistency and accurate quantification are required. Post-acquisition interrogation of selected data yields high quality quantification of target proteins comparable to targeted MRM analyses (Gillet et al., 2012). SWATH has only recently been introduced and therefore it has not been widely used in pharmacology research; reported applications include profiling of hepatic drug-metabolizing enzymes (Jamwal et al., 2017) and quantification of enzymes and transporters in pooled liver, intestine and kidney microsomes (Nakamura et al., 2016). Importantly, the utility of SWATH has recently been demonstrated in digital biobanking of tissue proteomic maps in health and disease (Guo et al., 2015).

6. Key pharmacology applications of proteomic data

The interaction between various intrinsic and extrinsic factors that affect patient populations can result in variability in the expression levels of PK-relevant proteins and PD targets, leading to variations in drug exposure and response profiles (Fig 2A). Proteomic methods are used to assess the effects of these factors, including age (Bhatt et al., 2019; Boberg et al., 2017; Prasad et al., 2016; van Groen et al., 2018), disease (Al-Majdoub et al., 2019; Billington et al., 2018; Margaillan et al., 2015; Prasad et al., 2018; Wang et al., 2016), ethnicity (Kawakami et al., 2011; Peng et al., 2015) and genetics (Bhatt et al., 2019; Peng et al., 2015; Prasad et al., 2013), individually or in combination, on protein expression profiles. Changes in abundance associated with perturbed systems relative to control are then used to predict effects on the fate of drugs (Fig 2B) (Bi et al., 2013; Ishida, Ullah, Tóth, Juhasz, & Unadkat, 2018; Prasad et al., 2018; Vildhede et al., 2014, 2018; Wang et al., 2016).

Ideally, measurement of the effects on abundance and activity of functional proteins should be carried out and used to achieve robust predictions; however, specific substrates and optimized functional assays are still lacking for enzymes and transporters, with the exception of several CYP and UGT enzymes (den Braver-Sewradj et al., 2017; Walsky et al., 2012; Walsky & Obach, 2004). Abundance is commonly used as a surrogate for activity; correlation between protein abundance and activity was demonstrated for various hepatic and renal drug-metabolizing enzymes, such as CYPs, UGTs, carboxylesterase 1, aldehyde oxidase 1, flavin-containing monooxygenases and sulfotransferases (Achour, Dantonio, et al., 2017; Chen et al., 2016; Fu et al., 2013; Knights et al., 2016; Margaillan et al., 2015; Ohtsuki et al., 2012; Venkatakrishnan et al., 2000; Wang et al., 2019; Xie et al., 2017). This was also demonstrated for certain transporters, such as P-gp and BCRP (Harwood, Neuhoff, Rostami-Hodjegan, & Warhurst, 2016; Kumar et al., 2015). *In vitro* measurements are therefore routinely extrapolated to *in vivo* activity (IVIVE) using scaling factors that rely on abundance measurements (Fig 2C) (Barter et al., 2007; Harwood, Achour, et al., 2016a). In addition to scaling, measuring the abundance of pharmacologically relevant proteins also allows evaluation of the sources of variability in activity rates; inter-individual variation is driven by variability in the level of expression, alterations in intrinsic protein activity, or a combination of these factors (Zhang et al., 2016). Below is a brief account of the main pharmacology applications of proteomic data. Each application requires a different level of proteomic analysis (absolute quantification, relative quantification or discovery/identification) as illustrated in Fig 3.

6.1. Physiology-based pharmacokinetic (PBPK) modeling and IVIVE

The use of PBPK models has now become firmly embedded in practices within the pharmaceutical industry and evidence from these models is used in different phases of drug development (Huang, Abernethy, Wang, Zhao, & Zineh, 2013; Jamei, 2016). PBPK modeling has gained wide acceptance with regulatory agencies (Rowland, Lesko, & Rostami-Hodjegan, 2015), with PBPK data being used in the labels of 21% of new drug applications approved by US Food and Drug Administration (FDA) in 2015 (Marsousi, Desmeules, Rudaz, & Daali, 2017). Modeling is commonly used for prediction of human pharmacokinetic parameters and evaluation of the effects of factors affecting a patient population, such as genetics and lifestyle (Heikkinen et al., 2015; Prasad, Vrana, Mehrotra, Johnson, & Bhatt, 2017). PBPK models are

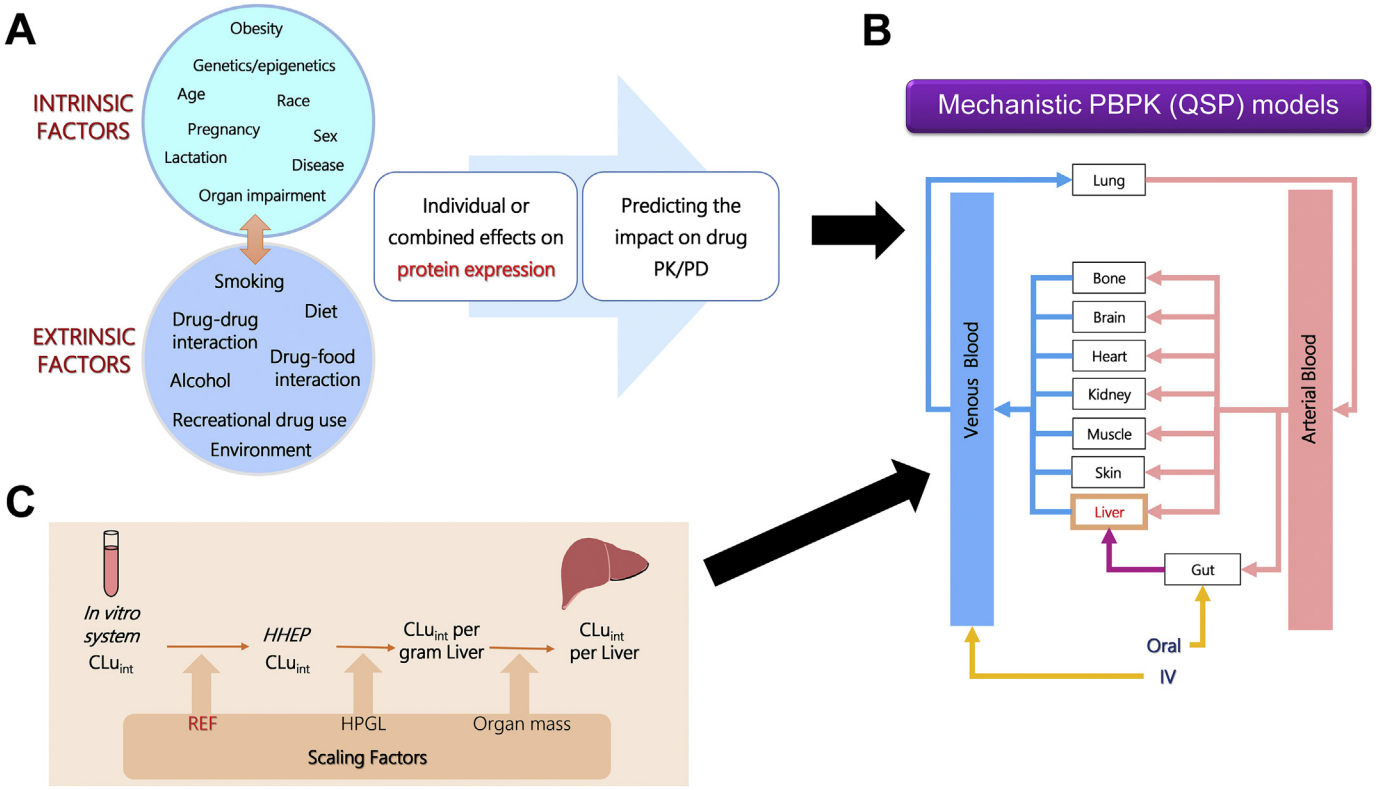

Fig. 2. The use of proteomic data in PBPK prediction of drug exposure. A. Several intrinsic and extrinsic factors can affect the abundance of proteins which in turn can affect drug PK and PD. B. Effects of intrinsic and extrinsic factors can be simulated using QSP (PBPK) models that incorporate physiological parameters (e.g. abundance) and drug data. C. The process of extrapolation from *in vitro* measurements in hepatocytes to the prediction of clearance in human liver; the process of IVIVE is used in combination with PBPK (or QSP) models (B) to predict drug PK (or PD) in a population of interest. Scaling factors used in IVIVE from hepatocytes are REF = the ratio of Abundance in tissue to Abundance in the *in vitro* system, HPGL and liver mass. Abbreviations: CLu_{int}, intrinsic clearance of unbound drug; HHEP, human hepatocytes; HPGL, hepatocytes per gram liver; IV, intra-venous administration; IVIVE, *in vitro-in vivo* extrapolation; PBPK, physiology-based pharmacokinetics; PD, pharmacodynamics; PK, pharmacokinetics; QSP, quantitative systems pharmacology; REF, relative expression factor measured using abundance data.

built by integrating drug profiles with physiological data, including blood flow, organ size, protein binding, and abundances of enzymes and transporters (Fig 2) (Jones & Rowland-Yeo, 2013). Various commercial and non-commercial platforms, e.g. Simcyp, GastroPlus, and PK-Sim, have facilitated the use of PBPK modeling (Kuepfer et al., 2016), but all require data describing protein abundance and population variability, and such data are still in short supply (Heikkinen et al., 2015). Key areas where PBPK models suffer from limited data include non-CYP and non-UGT metabolic pathways, extra-hepatic drug-metabolism and disposition, effects of differences in special populations (e.g. hepatically/renally-impaired, pediatric and geriatric patients) and inter-species variability. These limitations have started to be addressed in recent years mainly because of increased availability of (biopsy and surgical) tissue samples, advances in sample preparation methods and increased application of LC-MS proteomic techniques.

The use of IVIVE has extended the utility of PBPK modeling and made biosimulation more widely usable by linking modeling to *in vitro* studies using animal and human systems (Sager, Yu, Ragueneau-Majlessi, & Isoherranen, 2015). The application of IVIVE-PBPK requires integration of absolute abundance data in tissue relative to the *in vitro* system and system-specific scaling factors (e.g. microsomal protein content or hepatocellularity) with various patient-derived physiological parameters (Barter et al., 2007) to predict pharmacokinetic profiles and account for metabolic differences among specific populations (Rostami-Hodjegan, 2012). A recent systematic survey of the literature showed that the majority of PBPK models are used for the assessment of clinical pharmacokinetics and DDIs (Sager et al., 2015). Recently reported PBPK models that used proteomic data were developed for an array of applications, such as the prediction of variability in clearance (Harwood et al., 2016b; Kumar et al., 2018; Vildhede et al., 2018), variability in DDIs (Doki et al., 2018), impact of formulation (Johnson, Zhou, & Bui, 2014), effects of liver disease (Prasad et al., 2018; Wang et al., 2016) and kidney impairment (Zhao et al., 2012) on drug pharmacokinetics, and predicting drug kinetics in pediatrics (Jiang, Zhao, Barrett, Lesko, & Schmidt, 2013; Johnson et al., 2014; Ladumor et al., 2019), older patients (Polasek et al., 2013) and during pregnancy (Gaohua, Abduljalil, Jamei, Johnson, & Rostami-Hodjegan, 2012; Ke et al., 2013; Ke, Nallani, Zhao, Rostami-Hodjegan, & Unadkat, 2014). In addition to these applications, PBPK models represent a valuable tool for learning and internal decision making in the pharmaceutical industry as well as storing and integrating compound-specific information throughout drug discovery and development.

6.2. Quantitative systems pharmacology (QSP) models

Models with broader pharmacological applications include QSP models which represent new tools for drug development (Danhof, 2016), with several applications, including prediction of the effects of therapeutic agents, mechanisms of interaction between therapeutic targets and elucidating the biological processes underlying disease and resistance to drugs (Dimitrova et al., 2017; Kirouac, 2018; Kirouac et al., 2015). The US FDA has recently adopted the use of these models and the first case was the assessment of a novel parathyroid hormone replacement biologic (Peterson & Riggs, 2015). The use of QSP models for supporting new drug submissions is therefore expected to increase (Niu, Straubinger, & Mager, 2019). In particular, a promising application of QSP models is the assessment of phamcodynamic DDI potential by probing the mechanisms of interaction of a drug combination in the system and exploring the outcomes of target perturbations, as reported recently for the interaction between glibenclamide and the glucose-

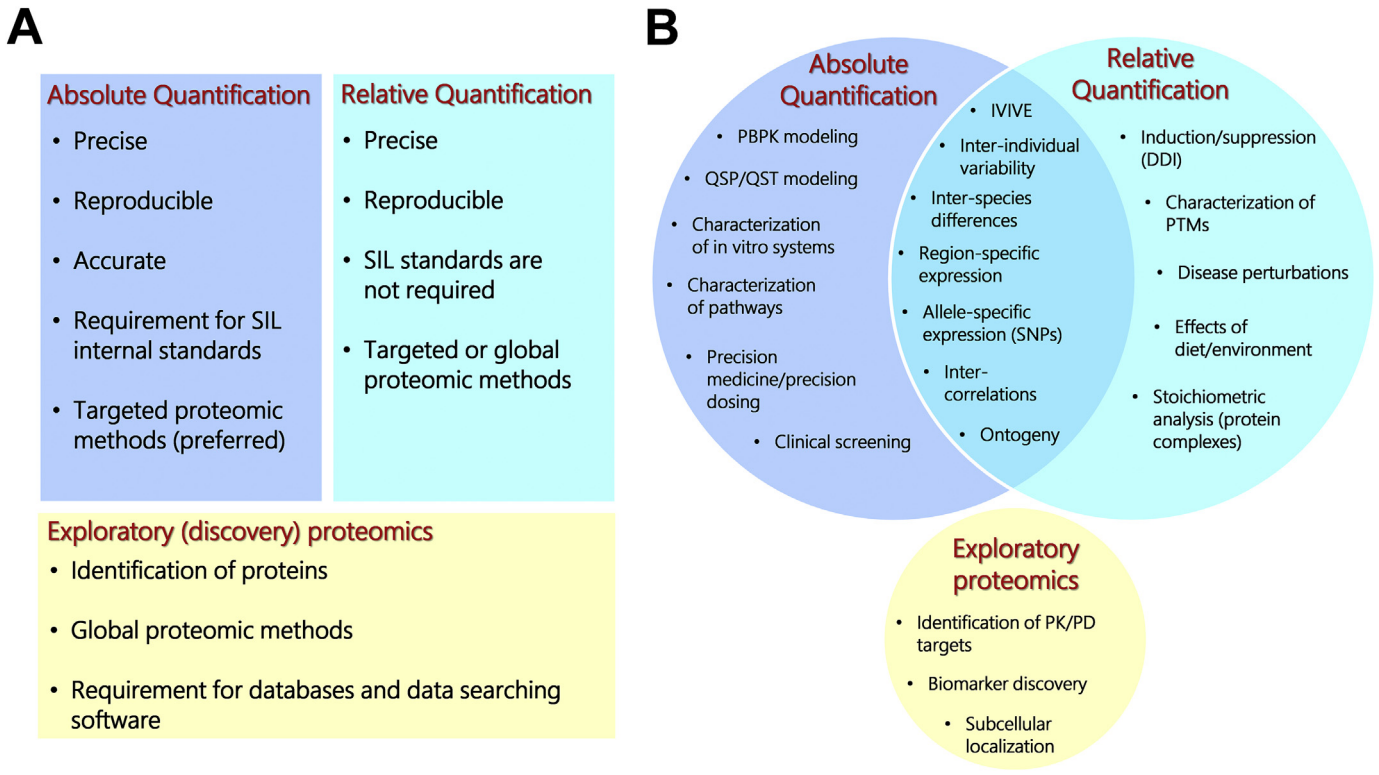

Fig. 3. The characteristics and applications of absolute quantification, relative quantification and discovery proteomic approaches. A. The requirements and characteristics of different levels of quantitative proteomic analysis. Absolute quantification requires assays that are accurate and precise; relative quantification requires reproducibility. B. Applications of data generated using absolute quantification, relative quantification and exploratory proteomics in translational PK and PD research. Several applications overlap between absolute and relative quantification. Abbreviations: DDI, drug-drug interaction; PBPK, physiology-based pharmacokinetics; PD, pharmacodynamics; PK, pharmacokinetics; PTM, post-translational modification; QSP, quantitative systems pharmacology; QST, quantitative systems toxicology; SIL, stable isotope label; SNP, single nucleotide polymorphism.

insulin-glucagon system in Type 2 diabetes (Choy, Hénin, van der Walt, Kjellsson, & Karlsson, 2013). The requirement for multi-omic data is emphasized for building pharmacology and toxicology models with the essential role of pharmaco- and toxico-proteomics in identifying and quantifying critical proteins in pathways affected by drug, chemical and environmental exposure (Wetmore & Merrick, 2004). This normally follows a strategy consisting of a discovery method followed by robust targeted quantification (Gillet et al., 2016). Proteomic data were previously used as the basis for developing QSP models to predict the effects of drugs, such as gemcitabine and birinapant in pancreatic cancer (Zhu, Shen, Qu, Straubinger, & Jusko, 2018) and 5-flurouracil in colorectal cancer (Hector et al., 2012).

6.3. Disease perturbation

Disease perturbation models are QSP models that aim to simulate disease progression and assess the effects of different drug regimens on a diseased population. Modeling disease perturbations requires relative abundance data for the diseased tissue compared to a healthy set of samples used as control. Disease-scale models have been applied to several disease states, including cirrhosis and different types of cancer. Cirrhosis is a disease of the liver that significantly affects drug metabolism and disposition and hence disease modeling can help with tailoring dosage regimens that are both safe and efficacious. Liver fibrosis generally leads to a reduction in expression of phase I and phase II enzymes (including CYPs, UGTs and sulfotransferases), and consequently, progressive decline in their abundance and activity is observed as the disease advances (Fisher et al., 2009; Hardwick et al., 2013). Proteomic evidence of changes in the abundance of CYPs, UGTs and other hepatic enzymes was reported in cirrhotic livers and was shown to be dependent on the cause of cirrhosis (Prasad et al., 2018). Phase I metabolizing enzymes are reported to be more influenced by disease progression than phase II pathways which can be attributed to shortage in blood supply reaching the scarred tissue (Yang et al., 2003). Incorporating proteomic data into disease-scale PBPK models has led to improved model performance in cirrhosis as reported for zidovudine, morphine (Prasad et al., 2018), repaglinide, bosentan, telmisartan, valsartan and olmesartan (Li, Barton, & Maurer, 2015; Wang et al., 2016).

Applications of disease models have also been highlighted for different malignancies, including breast cancer (Hodgkinson et al., 2012) and colon cancer (Hector et al., 2012). These models were mainly used to predict the prognosis in certain populations and assess the effect of anti-cancer regimens at different stages of the disease. Because of the difficulty in recruiting cancer patient populations in clinical studies and the ethical issues related to the exposure of healthy subjects to toxic anti-cancer drugs, PBPK models are better accepted in oncology drug development compared to other disease states (Yoshida, Budha, & Jin, 2017). There is currently a lack of abundance data in cancer, and LC-MS proteomics is set to address this gap by providing quantitative measurements of enzymes and transporters from biopsies and archived surgical samples (Prasad et al., 2017).

6.4. Protein inter-correlations

Inter-individual variation in drug PK and PD can largely be predicted by integration of known sources of variability, including demographic factors (e.g. age and ethnicity) and physiological parameters (e.g. blood flow, levels of enzymes and transporters) (Jamei, Dickinson, & Rostami-Hodjegan, 2009). *In silico* approaches, such as PBPK models, can simulate the interaction between different covariates, such as changes in enzyme/transporter abundance, and predict their effects on clearance and DDIs (Doki et al., 2018; Melillo, Darwich, Magni, & Rostami-Hodjegan, 2019). Considering the inter-correlation between the expression levels of pharmacologically active proteins, and indeed

between other physiological parameters (e.g. liver size and blood flow), can lead to more plausible parameter combinations when sampling from a population distribution (Tsamandouras, Wendling, Rostami-Hodjegan, Galetin, & Aarons, 2015). Multiplexed quantitative proteomics can measure multiple enzymes and transporters in individual biological samples simultaneously, allowing robust assessment of inter-correlations between these proteins (Achour, Barber, & Rostami-Hodjegan, 2014; Prasad et al., 2019). Due to the nature of correlation analysis, technical bias can in some cases lead to apparent relationships in protein expression and therefore caution should be exercised in order to use only verified biological inter-correlations in modeling applications (Heikkinen et al., 2015).

While various inter-correlations between drug-metabolizing enzymes and transporters have been confirmed both at the RNA (Izukawa et al., 2009; Wortham, Czerwinski, He, Parkinson, & Wan, 2007; Zhang et al., 2016) and protein levels (Achour, Russell, et al., 2014; Cheung et al., 2019; Couto et al., 2019; Mooij et al., 2016), the quantitative impact of such relationships on pharmacokinetic outcomes has only recently started to be explored, with models incorporating inter-correlations outperforming those that do not (Barter et al., 2010; Doki et al., 2018). It is expected that the use of more realistic combinations of physiological parameters will be widely practiced in PK and PD modeling and simulation (Melillo et al., 2019).

6.5. Precision dosing

Model-informed precision dosing (MIPD) aims to predict the right dose of a drug for a specific patient based on individual characteristics. This is expected to lead to improved efficacy and reduced toxicity and pave the way to individualized therapy (Darwich et al., 2017). This approach is most applicable to drugs with a narrow therapeutic index and for special populations, such as pediatrics, geriatrics and patients with hepatic and renal impairment (Polasek, Shakib, & Rostami-Hodjegan, 2018). Multi-omic approaches and recent developments in 'liquid biopsy' assays (Rowland et al., 2019) are expected to facilitate the construction of 'virtual twins' as a useful strategy to enable precision dosing. A 'virtual twin' is an *in silico* model that represents an individual patient, created by integrating system parameters (i.e. demographic, clinical and enzyme/transporter abundance data) from the patient in order to simulate individualized drug response (Patel, Wiśniowska, Jamei, & Polak, 2018). This requires collection of absolute and relative expression data (Polasek et al., 2018) measured in individual patients using innovative sampling techniques, such as the use of biofluids (Boukouris & Mathivanan, 2015).

6.6. Ontogeny

The process of growth and maturation is thought to be the main contributor to observed differences in drug PK profiles across the pediatric population age range and when compared to adult populations (Fernandez et al., 2011). For example, physiological changes, such as gastric pH and emptying and intestinal motility that occur from birth to adulthood affect the rate of drug absorption. This is particularly evident in neonates in which absorption is generally delayed (Batchelor & Marriott, 2015; Lu & Rosenbaum, 2014). In addition, the ontogeny of drug-metabolizing enzymes, such as CYPs and UGTs, and transporter proteins within the liver and other organs contributes to variable rates of drug metabolism and excretion (Badée et al., 2019; Bhatt et al., 2017, 2019; Boberg et al., 2017; van Groen et al., 2018), with consequences for toxicity and efficacy profiles (Batchelor & Marriott, 2015; Elmorsi, Barber, & Rostami-Hodjegan, 2016).

Current drug dosing regimens for pediatrics are based on allometric scaling from adult populations or reliant on local guidance and clinician experience because of lack of data from clinical trials (Calvier et al., 2017). Regulators are increasingly supportive of mechanistic PBPK models to inform drug labels in lieu of clinical trials in pediatric applications (Jones et al., 2015; Miller, Reddy, Heikkinen, Lukacova, & Parrott, 2019). There is still, however, a paucity of data to feed these pediatric models, in large part because pediatric samples are obtained opportunistically (Howard, Barber, Alizai, & Rostami-Hodjegan, 2018; Templeton, Jones, & Musib, 2018).

Despite the difficulties of sample collection, there is consensus that the abundance and function of the majority of enzyme and transporter proteins are comparatively low in fetal and neonatal samples, increasing at varying rates as a function of age toward adult equivalent levels (Badée et al., 2019; Chen et al., 2016; Cheung et al., 2019; Upreti & Wahlstrom, 2016). For example, CYP3A4, UGT2B7 and P-gp are present in small amounts in neonatal samples, increasing toward or surpassing adult equivalent levels by 1–3 years of age (Bhatt et al., 2019; Mehrotra et al., 2015; van Groen et al., 2018). Conversely, CYP3A7 abundance is relatively high in fetal and neonatal samples, decreasing rapidly toward adult equivalent levels within 1 year (Leeder & Meibohm, 2016; Mehrotra et al., 2015). Incorporation of ontogeny profiles with *in silico* models has led to useful pharmacokinetic predictions for several drugs, such as theophylline (Ginsberg, Hattis, Russ, & Sonawane, 2004), propofol (Michelet, Van Bocxlaer, Allegaert, & Vermeulen, 2018), tramadol (T'jollyn et al., 2015) and valproic acid (Ogungbenro & Aarons, 2014), in children.

6.7. Characterization of polymorphisms

Most drug-metabolizing enzymes, particularly CYPs, and transporters, such as organic anion transporting polypeptides, are polymorphic with a range of clinical consequences (Oswald, 2019; Zhou, Ingelman-Sundberg, & Lauschke, 2017). Various genetic polymorphisms are non-synonymous and can be characterized at the protein level, while polymorphisms occurring in the regulatory region of a gene can affect gene expression and mRNA stability in a particular tissue but do not result in modifications to the protein sequence. The effect of polymorphism becomes significant when it causes variability to an extent that necessitates a change in the administered dose of a specific drug (Gentry, Hack, Haber, Maier, & Clewell, 2002); a case in point is CYP2C9 polymorphism and its effects on the required dose of the anticoagulant warfarin. Our group has previously developed an allele-specific proteomic workflow that can distinguish different polymorphic variants of CYP2B6 (Achour, Russell, et al., 2014; Russell et al., 2013). Shi et al. (2018) showed applicability of this approach to UGT2B15 with the aim of elucidating the regulatory mechanisms of UGT expression. Although relative quantification is as applicable to studying polymorphisms as absolute quantification, this application requires accurate and reproducible assessment of the stoichiometry of target enzymes (or transporters), and therefore targeted proteomic methods that employ a QconCAT standard are especially suitable (Achour, Rostami-Hodjegan, & Barber, 2019).

6.8. Disease biomarker discovery

Identification of biomarkers assists in understanding the pathophysiology of a disease and its progression, as well as monitoring patient response during therapy (Hector et al., 2012; O'Dwyer, Ralton, O'Shea, & Murray, 2011). This is applicable not only to traditional drugs but also to testing the efficacy of new candidates and comparing them to already available therapeutic agents. Often more than one biomarker is necessary to characterize a disease state, where the synergy between several targets in the same (or related) disease pathway makes a composite test more effective than monitoring a single biomarker of disease (Russell et al., 2017). A rigorous discovery proteomics workflow should consist of a preliminary discovery phase using global proteomics, such as shotgun DDA or SWATH profiling, followed by verification or validation of target proteins using more quantitative targeted techniques, such as MRM or PRM. The settings of the targeted experiment will depend on information collected in the discovery phase (Prasad et al., 2019).

The initial step can be performed on a small set of well-characterized samples from patients with the relevant disease state relative to control with the aim of identifying differentially expressed proteins (Gillet et al., 2016; O'Dwyer et al., 2011). Global proteomics has led to the discovery of various diagnostic biomarkers, such as proteins related to resistance to cancer chemotherapy, and biomarkers for monitoring treatment (Russell et al., 2016; Srivastava & Creek, 2019). These biomarkers are normally associated with critical cell function pathways, such as survival, proliferation (Shruthi, Vinodhkumar, & Selvamani, 2016), apoptosis (Hector et al., 2012) and post-translational modification of proteins (Held et al., 2010). After conclusive identification of a set of biomarkers, targets are quantified in samples from different populations, such as patients at different stages of the disease, and a healthy cohort (Elschenbroich et al., 2011; Sjöström et al., 2015).

A promising application of global proteomics showed differences in expression profiles between Crohn's disease and ulcerative colitis, which are symptomatically very similar but require entirely different treatment regimens (Starr et al., 2017). In cancer, a wide range of signalling pathways can be perturbed, including the function of protein kinases and phosphatases, which can be monitored as disease biomarkers and targeted by novel drug therapies (Bhullar et al., 2018; Bollu, Mazumdar, Savage, & Brown, 2017). Recently characterized cancer biomarkers for the assessment of prognosis and for therapy-related considerations include HER2 for decision-making in cancer treatment (Kirouac et al., 2015), cAMP-CREB1 axis as a key mechanism associated with resistance to platinum-based therapy (Dimitrova et al., 2017), caspase networks associated with prognosis of colorectal cancer (Hector et al., 2012), Stathmin-1 in relation to cell migration in colon cancer metastasis (Tan et al., 2012), and protein Z as an early biomarker for the detection of ovarian cancer (Russell et al., 2016).

7. Recommendations for best practice in applying proteomic techniques

With the recent expansion in the use of proteomic techniques in clinical and pharmacology research, robust guidelines have become crucially required for choosing the most appropriate method for a specific application. The decision-making process tends to be complex and will depend on multiple factors including the biological question, the type of sample, the number of samples, the number of targets, and the available budget. Fig 4 shows a simplified decision tree intended to guide the choice of proteomic methods used for pharmacology applications. In the same line, a workshop was recently held by the International Society for the Study of Xenobiotics (ISSX), with the aim of reaching a consensus on the use of proteomics in translational pharmacology research. Various recommendations for the choice and application of different techniques were proposed but a general consensus was not achieved (Prasad et al., 2019).

Considerations for choosing a technique will generally differ for targeted and global proteomic methods. In targeted analysis, isotopically labeled standards are used to improve precision and accuracy of measurements and reduce bias caused by variations in sample preparation and matrix effects (Bhatt & Prasad, 2018). This is desirable when accurate quantification of inter-individual variability is required for QSP models and MIPD. Techniques recommended for these applications include MRM applied on triple quadrupole instruments and PRM conducted on higher resolution platforms, such as Orbitrap and Q-TOF instruments. Both methods can be used for multiplexed quantification and they offer a wide dynamic range, typically two orders of magnitude, and therefore spiking of standards should be guided by the range of targeted proteins. One of the main advantages of targeted analysis is possibly its unparalleled sensitivity achieved even in the presence of a complex biological matrix (Holman, Sims, & Eyers, 2012). Therefore, recommended practice is to quantify protein expressed at very low abundance in a targeted manner. MRM is currently the 'gold standard' in clinical and pharmacological research (Carr et al., 2014), and recent guidelines by the Clinical Proteomic Tumor Analysis Consortium (CPTAC) provides recommendations and standard operating procedures (SOPs) for the development, application and reporting of MRM assays (Abbatiello et al., 2017; Whiteaker et al., 2014). Large-scale cross-laboratory assessment of plasma proteins showed improved quantification when harmonized SOPs are followed (Abbatiello et al., 2015). Triple quadrupole instruments used for MRM are less expensive than higher resolution mass spectrometers and the use of scheduled MRM improves the reproducibility of the data and increases the number of peptides that can be analyzed in one experiment (Oswald, Gröer, Drozdzik, & Siegmund, 2013), thus reducing the cost and time of analysis. PRM methodology offers advantages in selectivity, resolution and sensitivity while requiring a lower level of method development compared to MRM (Peterson et al., 2012). Orbitrap and Q-TOF instruments tend to be expensive but they represent versatile platforms capable of targeted (PRM) and global analyses (Peterson et al., 2012; Schilling et al., 2015).

Targeted techniques rely on the use of labeled standards and the choice of suitable standards depends on the type of experiment and available budget and expertise. Isotope-labeled internal standards tends to be expensive, but they provide better quality quantification (higher precision and accuracy) than label-free methods. AQUA peptides are ideal for screening applications where a small number of proteins (< 10) are monitored in a large number of samples. QconCATs are more applicable when higher numbers of proteins are targeted and for applications that require strict stoichiometry of standards, such as allele-specific proteomics (Achour et al., 2019; Shi et al., 2018). QconCAT standards have the advantage of sustainability and transferability across laboratories (Russell et al., 2013); a plasmid can be shared by different groups with access to protein expression facilities. We have previously developed a cost-benefit framework to assess the use of quantitative proteomic methods based on cost and application (Al Feteisi, Achour, Barber, & Rostami-Hodjegan, 2015). This assessment showed that the high cost of PSAQ standards hinders their application when a considerable number of proteins are targeted.

For applications that aim to identify novel proteins or quantify a large number of targets (> 100 proteins), the method of choice is global proteomics. Shotgun global proteomics, in conjunction with the TPA approach, can be cheaper than targeted methods because they do not require the use of labeled standards. This method is applied with Q-TOF and Orbitrap instruments and has a wide range of hypothesis-generating applications, including proteome-wide analysis, assessment of disease perturbations and biomarker discovery. Data-independent methods, such as SWATH, offer increased depth of analysis and quantitative reproducibility (Gillet et al., 2012), making them very suitable for generating protein network data for systems pharmacology applications. Their use is however still restricted to core facilities, and sophisticated bioinformatics tools are required for data analysis and interpretation (Distler et al., 2014; Röst et al., 2014). A combined discovery-quantification strategy is recommended when characterizing a novel target or disease pathway (Gillet et al., 2016). This requires using global analysis (e.g. SWATH) on well-defined (disease and control) samples followed by targeted (MRM or PRM) quantification.

The concept of a 'proteomic map of disease' has recently been proposed (Guo et al., 2015; Xu et al., 2019), supported by highly reproducible sample preparation and global proteomic workflows. We recommend that major academic centers should conduct harmonized efforts to generate and share similar proteomic maps in health and disease for available biopsy and surgical samples from different tissues, as demonstrated recently (Uhlen et al., 2015). This will likely require the use of highly reproducible methods capable of wide proteome coverage, such as SWATH-MS (Gillet et al., 2012), and these digital maps can be interrogated retrospectively by various groups for future applications.

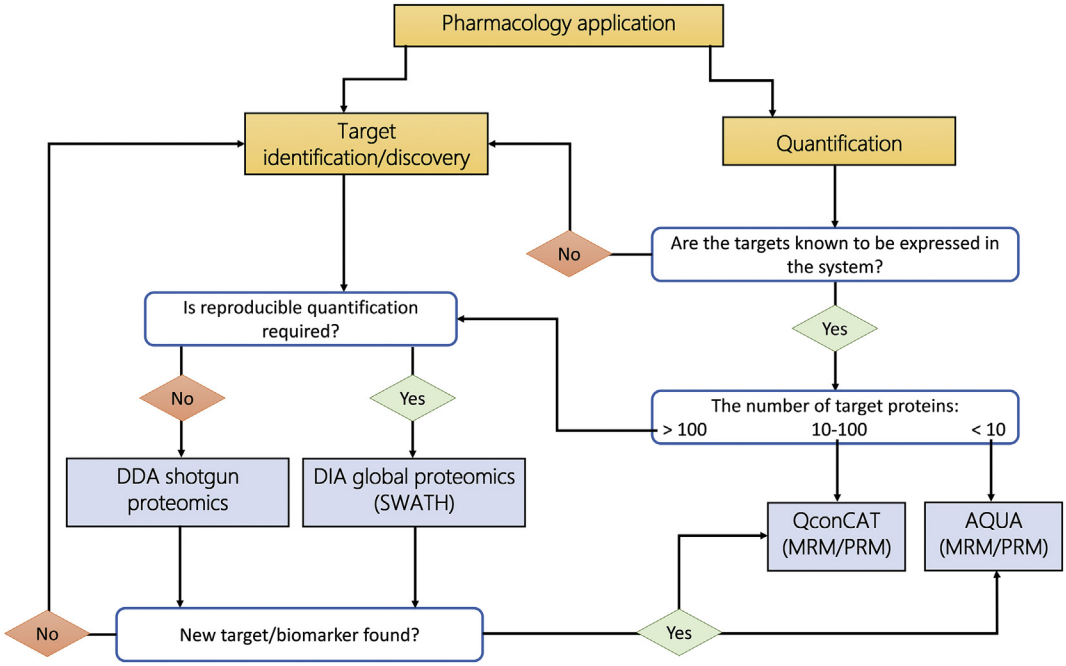

Fig. 4. Decision tree for choosing suitable proteomic techniques intended for pharmacology applications. A typical number of samples (~30) is used as input for the decision tree. The application can be hypothesis-driven and focused on quantification or hypothesis-generating and intended for discovery. If the application is focused on discovery, global proteomics is most suitable, with preference for data-independent acquisition when reproducible quantification of differential expression is required. When a target or a biomarker is discovered, more accurate quantification is achieved with targeted proteomics. If the target proteins are known to be expressed in the system and are well-defined, targeted proteomics is preferred. If the number of targets is small (< 10), AQUA-based methods (in conjunction with MRM or PRM techniques) are cost-effective. When the number of targets is higher (10-100), QconCAT methodology is preferred. Quantification of larger numbers of targets (> 100) and characterization of proteomes is better achieved using global proteomics. Orange boxes denote applications and blue boxes represent proteomic methods.

8. Conclusion

Quantitative proteomic measurements can make a significant contribution to the advance of quantitative systems pharmacology and can be relatively quickly translated into the clinic, where they directly benefit patients. These measurements are powerful, providing selectivity and sensitivity unparalleled by other protein-level techniques. The disadvantage of the unparalleled sensitivity is that independent orthogonal verification of a measurement is often challenging. Further, the cost of these experiments and small sample sizes preclude extensive sample sharing and cross-laboratory analyses. Prasad et al. (2019) have highlighted the difficulty in obtaining consensus as to appropriate protocols for different measurements, especially as the most thorough approaches are beyond the budgets of many laboratories.

We can however make a number of broad observations. Firstly, targeted methods are preferred where a specific, poorly expressed set of proteins is to be quantified, whereas global methods are better adapted to gaining a general picture of the functional proteome in a cell. Secondly, while there is merit in terms of accuracy in analyzing unfractionated samples, the loss of precision and sensitivity compared with the use of fractions is often critical. Thirdly, neither QconCAT proteins nor AQUA peptides are ideal as standards for targeted proteomics; QconCATs are favorable where large numbers of similar samples are to be analyzed for several proteins, whereas AQUA peptides are effective for small numbers of target proteins. When a decision is made, the minimal requirement is that the use of a particular quantitative proteomic technique should be 'fit for purpose'. Ultimately, the selected method and the level of proteomic quantification will have a substantial impact on the quality and validity of model-informed predictions.

Declarations of Competing Interest

The authors declare that there are no conflicts of interest.

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