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Proteomic studies of bone and skeletal health outcomes

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ABSTRACT

Proteins are an essential part of essentially all biological processes, and there is enormous variation in protein forms and concentrations that is not reflected in DNA or RNA. Recently there have been rapid advances in the ability to measure protein sequence, modification and concentration, particularly with methods based in mass spectrometry. Global measures of proteins in tissues or in the circulation provide a broad assessment of the proteome that can be extremely useful for discovery, and targeted proteomic measures can yield specific and sensitive assessments of specific peptides and proteins. While most proteomic measures are directed at the detection of consensus peptide sequences, mass spectrometry based proteomic methods also allow a detailed examination of the peptide sequence differences that result from genetic variants and that may have important effects on protein function. In evaluating proteomic data, a number of analytical considerations are important, including an understanding of missing data, the challenge of multiple testing and replication, and the use of rapidly evolving methods in systems biology. While proteomics has not yet had a major impact in skeletal research, interesting recent research has used these approaches in the study of bone cell biology and the discovery of biomarkers of skeletal disorders. Proteomics can be expected to have an increasing influence in the study of bone biology and pathophysiology.

Key terms and abbreviations in proteomic technology.

Abbreviation	Term	Relevance to proteomic studies
MS	Mass spectrometry	Analysis platform utilized for peptide/protein detection and quantification
MS/MS	Tandem mass spectrometry	Refers to using one parent MS scan to detect peaks and a second MS in which a single peak is isolated and fragmented for identification.
LC	Liquid chromatography	Separation approach often coupled with MS that provides increases in peptide detection
m/z	Mass to charge ratio	Primary measurement unit that distinguishes detections of peptide/proteins in MS
DDA	Data dependent acquisition	MS/MS analysis approaches that specify whether specific m/z values (DDA) or wide ranges (DIA) are isolated prior to fragmentation in global analysis.
DIA	Data independent acquisition	
MRM	Multiple reaction monitoring	MS-type methods used for targeted peptide measurement. Quantification is more precise than in global proteomic methods like MS/MS.
PRM	Parallel reaction monitoring	
SRM	Selective reaction monitoring	
CID, ETD, H-CD, ECD	Collision induced dissociation, electron-transfer dissociation, higher energy collisional dissociation, and electron capture dissociation	Fragmentation approaches used in MS/MS analysis platforms
SILAC	Stable isotope labeling by amino acid in cell culture	Approach used to incorporate heavy isotopically labeled amino acids into proteins to improve MS signal quantification
TMT, iTRAQ	Tandem mass tag, isobaric tag for relative and absolute quantification	Labeling approach to improve quantification of peptide detection in MS
SWATH-MS	Sequential Window Acquisition of All Theoretical Mass Spectra	A common DIA analysis method
SEQUEST, MASCOT, MSGF+		Fragmentation spectra search programs that identify peptide sequences
MAXQUANT		Comprehensive quantitative MS program for data processing of MS results

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1. Introduction

Proteins mediate virtually all cellular processes. Consequently, disease almost always manifests at the protein level, and modulation of protein function can have therapeutic effects. While it is often assumed that mRNA levels reflect the abundance and activity of their respective proteins, the ability to predict protein levels from transcript abundance is modest at best [1–3]. Systematic quantification of proteins shows that transcript abundance is an imperfect predictor of protein level either in steady state or in response to perturbation [2]. For example, a recent analysis of mRNA and protein over the course of fracture healing in mice demonstrated that only 88% of protein and transcript profiles were concordant (i.e., both up- or both down-regulated) [4]. As a result of transcription and splice variants, post-translational modifications, and variation in processing and degradation, the proteome is large and heterogeneous. For instance, the proliferating *Schizosaccharomyces pombe* cell contains ~60 million protein molecules [5]. The numbers of individual cellular proteins probably dwarf the number of genes and genetic modifications. Moreover, regulatory events critical to homeostasis are likely to be found primarily at the post-translational level involving alterations in protein configuration, abundance or function [6]. Proteomic measures are essential for understanding biological events, the analyses of biologic systems, and the development of diagnostics and therapeutics. Therefore, describing and understanding the function of the proteome is a central and fundamental challenge of biology.

In contrast to the increasingly well described human genome, the human proteome is still inadequately understood. But proteomics has recently moved into the fast lane [7]. Previous methods involving gel electrophoresis or antibody-based detection have given way to much more effective and high-throughput approaches. Mass spectrometric (MS) methods enabling precise protein measurements have evolved quickly and have tremendous promise for specific, reproducible and quantitative measurement of the levels of peptides, proteins, and their modified forms [8]. These increasingly accessible large-scale, MS-based technologies enable the evolution of analysis from single proteins to whole proteomes, from single-cell studies to tissue-level analysis and from static to dynamic measurements. Other high capacity measurement approaches, such as aptamer-based assays, have also been developed to enable broad assessments of the proteome [9]. Combining improved computational proteomics, instrument performance, sample preparation methods and assay design allow for robust quantification of a large fraction of endogenous proteins and their various modifications in tissues, plasma or serum [2].

Pipelines for large scale discovery proteomics have been used in pioneering biomedical studies [2,5,10] and are extremely attractive for hypothesis-free exploration of disease biomarkers [5,10,11]. Moreover, tools for understanding the proteome are improving the usability of these large datasets. The Human Protein Atlas, www.proteinatlas.org, a systematic exploration of the human proteome using antibody-based reagents, is a unique effort toward characterizing all human protein-coding genes [12]. It has developed into a knowledge base that includes proteomes of tissues [13], cells [14] and cancers [13]. It benefits a broad range of protein centric researchers enabling searching for any human protein for fundamental localization information at multiple levels. The SRMAtlas, www.srmatlas.org, is a compendium of highly specific proteomic assays that enable quantification of 99.7% of the 20,277 annotated human proteins [15]. It is primarily useful for proteomic researchers and others interesting in developing or utilizing advanced targeted protein sequence assays within their research. On a complex scale, the BioPlex project, bioplex.hms.harvard.edu, begins to define the architecture of protein communities and disease networks. It includes protein interaction networks and co-complexes based in > 25% of human protein-coding genes, with > 56,000 candidate interactions [16]. It is beneficial for those focusing on protein interactions and network associations which can help provide information on the underlying mechanisms of a researchers specific study model.

Here we will briefly review proteomic measurement approaches with a focus on mass spectrometry, consider some data analysis considerations when using proteomics, and summarize the emerging use of proteomics for skeletal research. Other reviews on proteomics and bone research have also highlighted similar issues [17,18].

2. Current MS-based proteomic technologies and approaches

Here we will discuss the available MS-based technologies for proteomic analyses and examine the study designs. We hope to convey the potential usefulness of these approaches for skeletal investigation and in doing so help facilitate their incorporation into clinical and basic research studies. We provide a general overview of the current proteomic approaches that are most commonly used to examine biological specimens in mass spectrometry laboratories.

Proteomic technologies and approaches are agnostic regarding the biological sample being studied; hence, general proteomic approaches are applicable to most specimens relevant for bone and skeletal focused research, including clinical biofluids, tissues, and isolated cells or cell culture based in vitro systems. Bone specific protein sample isolations [19–21], exosome isolations [22,23], and secreted/extracellular matrix vesicle isolations [24,25] have been modified for proteome analysis, and any future isolation or enrichment scheme is likely to be modifiable as well.

An exhaustive summary of all possible approaches is beyond the scope of this review, as the field of MS based proteomic analysis is large and continually evolving. Advances are driven by the need for greater measurement sensitivity, higher throughput, and greater accuracy and robustness in quantification. Most research institutions now have a dedicated MS core (or research group) that investigators can consult regarding their specific MS-related research questions.

Current proteomic approaches can be generally divided into two major efforts, termed "global" or "discovery" proteomics and "targeted" proteomics. Global proteomics is designed to examine as much of the proteome as possible in a sample. It can involve analyses of either peptides or intact proteins. Liquid chromatography (LC)-MS based intact protein ("top-down") analysis has made tremendous advances in the past several years in both analysis sensitivity, and informatics support [26,27]. However, initial proteome comparisons are still likely to be accomplished using peptide-level or "bottom-up" strategies. This approach involves trypsin digestion of the proteins in a sample and the identification of the resulting peptides, from which protein level information is inferred using informatics methods. We will focus primarily on the more traditional LC-MS analysis driven by peptide-level isolation and detection.

On the other hand, "targeted" proteomic approaches are designed to assess a limited number of specific peptides or proteins and most often involve selective reaction monitoring (SRM) or multiple reaction monitoring (MRM) MS-based experiments. Compared to global measurements, targeted methods improve ion signal and hence quantification. Parallel reaction monitoring (PRM) is another MS-based approach in which a high mass resolution MS platform is used to multiplex and characterize the final fragmentation ions [28]. Targeted measurement methods that are not MS-based include traditional antibody-based immunological assays and newer aptamer-based assays. Aptamers are small (usually from 20 to 60 nucleotides) single-stranded RNA or DNA oligonucleotides able to bind target proteins with high affinity and specificity, thus enabling sensitive and specific detection.

2.1. Global proteome analysis

Current global proteomic analysis is based upon combining LC separation of the peptides in the sample with electrospray ionization into a MS platform that fragments peptide ions to obtain MS/MS spectra for peptide identification and quantitation. These steps are accomplished

without prior knowledge of specific peptide or protein sequences expected to be present in the samples. Current hybrid MS instrumentation (those instruments that can both generate MS/MS fragmentation and make accurate mass measurements for quantification) is now fairly common. The speed of analysis is generally a function of the LC component and can range from 10 min to several hours; a typical analysis time is 30 to 120 min per sample. Due to the diversity of current MS instrumentation, there are almost endless protocols that can be followed, each with their own biases and strengths. These include data-dependent acquisition (DDA) and data-independent acquisition (DIA) approaches [29] as well as various fragmentation strategies (CID, ETD, HCD, ECD, among others – see Key Terms for definitions). In general, the goal is to obtain in-depth detection, identification, and quantification of the peptide or protein components of the sample.

In terms of biological analysis, global proteomics are the only truly unbiased protein-based analysis approach. That is, proteins can be identified and their relative abundances quantified without any prior expectation. However, there are several key aspects that must be considered.

2.1.1. Sequence-specific databases

A well sequenced species-specific database containing protein sequences of interest must be available so that peptide sequences found in the sample can be matched to known sequences. There are now extensively annotated human and mouse databases available, as well as sequenced versions of most relevant animal model systems, which also facilitate data matching to genomic measurements. As this approach is sequence specific it can capture expressed isoforms and sequence variants only as long as they are included in the reference database. For instance, in the proteomic studies of vitamin D binding protein (VDBP) described below [30,31], genetically determined protein variants were not detected in consensus sequence-based protein databases (also see subsequent discussion on data missingness). It was necessary to specifically identify variant VDBP sequences in the proteomic datasets and to design SRM assays to quantitate them.

2.1.2. Breadth vs depth

The broad, quantitative information obtained in global analysis can be very informative, but as the focus is on breadth of coverage and accuracy of identifications, the ability to detect low abundance proteins generally suffers compared to targeted MS approaches (SRM and MRM) and other protein quantification methods that include protein-specific enrichment approaches, e.g., ELISA or aptamer methods [32]. Poorer sensitivity in global discovery proteome analysis is in part because of the very large dynamic range of complex protein mixtures (e.g., serum or plasma) that greatly complicates the detection and quantitation of all peptides present. Low signal intensity of a detected but lower abundant peptide is inherently more variable in measurement, hence researchers often use extensive pre-fractionation approaches and enrichment methods to reduce the complexity and dynamic range of biological samples [33,34], allowing more peak detection, greater signal to noise measurements and more accurate quantification. The downside to such approaches is that this greatly increases the analysis time for a particular sample or set of samples. Depending upon the sample size of a study, there is often a balance between the level of sensitivity or depth of coverage required and the time and resources devoted to proteomic data collection. As discussed below, proper experimental designs are critical in ensuring the results will address the biological question at hand. Confident quantitation of protein or peptide concentrations is challenging. Numerous approaches that at one time were extensively used to help quantify proteomic outputs (e.g., ICAT, O18 labeling, spectral counting) have been displaced by more robust methods through improvements in both instrumentation and informatics. Current approaches can largely be separated into either 1) standard-free (label-free) approaches that yield relative abundance or protein/peptide mass and 2) experiments that incorporate some form of labeling

(usually as isobaric labeled compounds) to multiplex samples and increase fractionation/coverage as well as provide a more controlled quantification comparison. In cell based studies it's also possible to take advantage of the incorporation of stable isotopes into amino acids for comparative quantification (i.e., SILAC).

2.1.3. Label-free quantification

Label-free quantification uses the direct MS peak intensity information from the detected peptide (normally the area under the peak distribution over its elution time) from which a quantitative value is assigned. It is generally based upon a high-resolution capture of the parent peptide peak intensities. Benefits of this approach are that it is fairly straightforward, with multiple analysis platforms available to generate such data, and it does not require much post-processing beyond standard normalization approaches. The downside to label-free quantification is that it does not yield specific concentration measures (since there is no "standard"). Rather, relative abundance values are the primary result. Also, as a direct intensity measurement it is subject to variations in instrument sensitivity, electrospray ionization, and sample loading. Good reproducibility requires strict study design, blocking, and downstream analysis. This is especially true when analysis of larger sets of clinical samples is planned, when analysis time can stretch into several days or weeks and the MS instrument must be cleaned within sample sets.

2.1.4. Labeling strategies

As an alternative approach, labeling strategies help alleviate many of the issues with label-free quantification. Popular isobaric reagents (TMT [35] and iTRAQ [36]) allow for both multiplexing (4, 6, 8, or 10 multiplexed sets) and easier merging of quantitative information after fractionation, reducing overall instrument time while providing deeper, more quantitative coverage. As each sample is labeled with a unique isobaric tag prior to mixing, during MS/MS fragmentation this unique tag is released, providing a specific quantitative peak in the low mass range (m/z –mass to charge ratio) of the MS/MS spectra. This region is generally devoid of actual peptide fragmentation leaving only a series of quantifiable peaks that correlate with the various unique tags. These unique peak intensities are often called "reporter ions" as they are used to report the number of ions detected for a specific fragmented tag. The intensities of these reporter ion channels are then used to compare between samples within the same labeling experiment, and across different experiments (made easier if one channel is used as a universal pooled control). As comparisons are done by ratio and within each spectra, variations caused by absolute signal intensity values are minimized. There are minor issues involving label compression and channel overlap [37,38] which can be addressed, but the overall downside to such an approach is primarily the extra cost of the commercially available reagents and the extra time associated with preparation of the samples. For many laboratories, this has become the primary approach for global quantitative analysis.

2.1.5. Informatics

Informatics support is critical for proteomics. Informatics methods for global proteome analysis vary somewhat depending upon the approach, but overall relies on using a MS/MS spectra matching software (SEQUEST, MASCOT, MSGF+, MAXQUANT) to make identifications followed by quantification. DIA methods such as SWATH-MS (Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra) require more nuanced searching as the MS/MS fragmentation data is multiplexed and requires extraction of the appropriate fragment ions [39]. There are multiple approaches on inferring protein quantification based upon peptide level abundance, ranging from simple summing of all reporter ion intensities values for labeled studies to more complex programs utilizing direct peptide intensity information which attempts to mitigate the variations in peptide abundances within a protein [40–42].

Table 1
Proteomic study purposes and design choices.

	Proteomic study purpose		
	Prediction or classification for clinical utility	Association or classification for biologic insight	Classification for novel subtype discovery
Example study	BMD loss or hip fracture [57]	Osteoclast biology [83]	Colorectal cancer subtypes [84,85]
Sample source	Human population-at-risk	Human biopsy, animal model, or cell line	Human heterogeneous case mix
Sample type	Easily accessible, like serum or plasma	Biologically relevant, like bone tissue or osteoblasts	Accessible during diagnosis, like through blood sample or biopsy
Analytic goal/approach	Portable marker set to optimize prediction or classification	Sufficient marker set to describe biologic process	Portable marker set to optimally separate clinically meaningful classes
Proteomic technology	Global and targeted	Global	Global and targeted

2.2. Targeted analysis

Targeted analysis refers to assays that are designed to detect and quantitate specific peptides. While global proteomic methods provide information on relative peptide or protein abundance, targeted methods can yield definite measures of concentration. They focus on known peptide sequences or m/z values to quantify their abundance more accurately. Targeted proteomics by single reaction monitoring (SRM) was named method of the year in 2013 (Nature Methods) [43]. To date, there has been little targeted MS analysis performed in bone research, representing a unique opportunity to expand this analytical approach into this field of research. General MRM/SRM approaches rely on prior identification of a peptide sequence which is targeted in a LC-MS/MS experiment through the addition of heavy labeled peptide standards of the same sequence. The instrument of choice is a triple quadrupole MS which excels in the additional isolation and quantification of a transition or MS/MS fragment for quantification. The additional isolation of the transition provides a greater level of sensitivity due to reduced background noise, and coupled with observation of the heavy labeled peak, better quantification as the spiked standards serve as an internal reference [44, 45]. Though levels of detection are again dependent upon the overall abundance of the peptide/protein of interest, there are multiple methodologies which serve to augment the peptide signal of interest, most relying on either depletion of more highly abundant proteins, a common approach in plasma/serum studies [46], and/or addition of initial fractionation of the sample (for instance, via liquid chromatography) to reduce complexity of each fraction [47]. Further, experiments can be highly multiplexed with up to several hundred target sequences examined in a single analysis. If coupling a targeted study with a high mass resolution MS platform, quadrupole time-of-flight (QTOF) or orbitrap MS, then PRM can be used to assay across multiple targets by utilizing the high mass accuracy for the detection of both the precursor and fragment ions [28].

As the analysis is sequence specific and highly multiplexed, the clinical utility of such assays is becoming evident [45,48]. Targeted assays can be multiplexed to create biomarker panels that can outperform individual biomarkers and are becoming more prominent in the clinical arena [49,50]. Opportunities include the capability to target specific protein variants and isoforms, difficult to target using conventional immunoassay-based technologies. This was clearly seen in the example of VDBP (see below), where sequence-specific SRM assays were developed for multiple sequence variants with implications for racially diverse populations [30,31]. Despite these advantages, biomarkers or biomarker panels using proteomic methods have been little used in skeletal research or clinical applications.

2.3. Detection and analysis of protein modifications

For both global and targeted methodologies, a significant benefit of proteomic analyses is the ability to directly identify and quantify protein/peptide modifications, ignored by genomic studies and only previously obtainable from difficult to develop immunoaffinity assays. The most popularly targeted post-translation modification is phosphorylation, directly relevant to identification of pathway-based signaling networks which often are not measurable by protein abundance but by site-specific phosphorylation level. Multiple additional protein modifications have been detected and identified in MS studies (e.g., acylation, glutathionylation, S-nitrosylation, and methylation), but overall they are 1) in much lower abundance than unmodified protein portions, and/or 2) generally require selective enrichment or additional separations to robustly detect and quantify them (e.g., ubiquitination or glycosylation). Regardless of approach, both global and targeted studies can be designed to identify multiple modifications, particularly in regard to phosphopeptide enrichment and analysis. Targeting site specific phosphorylation events through SRM studies has proven to be exceedingly useful [51,52], augmenting other traditional molecular biology techniques such as site directed mutagenesis. Such studies are directly relevant to osteoblast cell culture studies, as well as bone tissue extracts.

2.4. Analysis strategies

As global and targeted proteomic approaches represent different strengths, they have been commonly utilized in complementary roles in overall analysis strategies (Table 1). Unbiased global analysis is often used as the initial discovery technology that provides a limited list of targets from which further validation studies are initiated [53]. The goal here is to be inclusive of potential targets, particularly those which appear biologically interesting. As the global analysis can lack quantitative accuracy, more liberal feature selection criteria can be employed, knowing that more robust quantitative values can be obtained in subsequent targeted validation studies. Targeted analyses are intended to quantify specific peptides, and are thus best used in hypothesis-based studies.

These two proteomic methods (global and targeted) are often used together to discover and then validate experimental findings. Once candidate peptides of interest are identified and parsed based upon biological interest or statistical significance using global (discovery) proteomic approaches, targeted SRM-based studies can be designed for more accurate quantification and validation of the initial discovery results, including multiplexing of appropriate peptide targets in each SRM assay. This type of discovery (with global proteomics), followed by validation (with targeted proteomics) design can also be used to identify biomarkers initially detected from discovery proteomics in a tissue source with subsequent targeted evaluation in a more accessible sample type, such as serum, urine or cerebrospinal fluid. Though not all tissue-specific differentiating proteins are secreted or shed into the extracellular space and into circulation, using sensitive, follow-up targeted studies provide an opportunity to quantify proteins or peptides in blood or other fluids.

2.5. The importance of genetically determined protein sequence variation

The targeted proteomics approaches mentioned above that use

antibodies or aptamers are powerful, as they can survey a large number of proteins, including those present at low concentrations, in large populations. A potential problem with these analyses is that the probes (antibodies or aptamers) are usually designed to recognize consensus peptide sequences. However, one of the obvious complexities of the proteome is that genetic variation is reflected in similar peptide sequence variation. Sun et al. reported the considerable genetic control of the human plasma proteome and intermediate molecular pathways that connect the genome to disease using a Mendelian randomization approach [54]. Probes (antibodies or aptamers) designed for consensus peptide sequences may fail to recognize, or recognize with different affinities, non-consensus proteins. A value of MS-based proteomic analyses is the ability to determine and quantify individual peptide sequence variation. This may have enormous utility. Examples are the studies we and others performed to examine the proteomics of vitamin D binding protein (VDBP) [30,31,55]. Stimulated by a report that there were racial differences in total 25OHD levels because of differences in VDBP levels (determined by immunoassay), we used proteomic methods to determine the serum VDBP sequence variation that resulted from racial differences in VDBP genetics. In fact, genetically driven protein sequence differences were obvious, and in turn adversely affected the performance of the immunoassay methods initially used to determine VDBP concentrations. It's likely that similar genetically determined protein sequence variation affects assays of other proteins, as well as their functions. These observations mean that plasma (or serum) is a sample type fertile for proteomic exploration, but ideally it should be interpreted in light of the genomic variation that complicate these studies, and the ability of MS-based proteomic methods to specifically quantitate variation in protein sequence may be enormously important. Furthermore, proteomic analyses are capable of assessing post-translational protein modifications (e.g., chemical modifications of protein termini and amino acid side-chains, phospho-, S-nitrosyl, carbonyl and D-aspartic acid formation, conversion to D-aspartic acid) [56] that may also affect both function and detection by other assays.

3. Considerations for data analysis in proteomic studies

Fulfilling the promise of novel and relevant discoveries through proteomics requires consideration of several analytical features, some of which are shared with other omics approaches while a few are unique to proteomics. Like other omic studies, proteomics requires careful control for multiple testing and validation of statistical associations to avoid false-positive reports. Additional issues for proteomic studies are the choice of sample- or tissue-type to be analyzed and the high proportion of missing values, which can sometimes be informative. As in early genomic studies, most recent proteomic studies have lacked replication cohorts, and investigators have turned to existing genomic and transcriptomic data sets to corroborate findings and prioritize protein and pathway associations. The design of initial studies, their analysis, and subsequent research depend on the primary research question, and the following section uses three categories of question as a framework for discussion (Table 1). In addition, some data analysis issues are particularly germane for proteomic studies.

3.1. Analytic considerations: missing data

In human studies that use high throughput proteomics technology, the number of proteins that are identified in *any* individual (thousands) dwarfs the number confidently identified in *all* individuals (hundreds) [23, 57–59]. In practice, those proteins with insufficient representation (e.g., identified in < 50% of individuals in the study) are excluded from the analytic pipeline. For proteins identified in only a subset of individuals, power is usually insufficient to detect associations with outcomes. Unfortunately, this loss of information, which varies across studies, limits comparison and replication of results in independent samples. Moreover, protein missingness cannot be assumed to be

random and therefore can introduce bias into subsequent association analyses. In our proteomic analysis of VDBP, we observed that men with African ancestry were missing values for VDBP peptides after routine peptide sequence matches to human protein databases that were primarily linked to Caucasians. Using genomic predictions of alternate sequences, we were able to correctly match African-ancestry to unique VDBP peptide sequences to complete the profile of VDBP across racial-genotypic variation [31]. This analysis used a single protein to demonstrate the pitfalls of ignoring missing peptide abundance values and the utility of searching for variant peptides in order to complete proteomic profiles in diverse populations. However, the methods and computational power to systematically piece together the proteomic profiles of each person's sample based on his or her genetically predicted sequence variants are not yet available.

3.2. Analytic considerations: multiple testing and replication

Most proteomic studies for skeletal phenotypes have analyzed individual protein associations with outcomes, followed by protein-set enrichment analyses to identify biologic processes or pathways of particular relevance [60]. Protein counts are usually in the hundreds, and a false-discovery rate adjustment is commonly used to avoid type I error. To summarize multiple peptides into an overall protein-level association, we have used a meta-analytic technique that yields a single "meta-analytic standardized fold change" to compare protein abundance across levels of the phenotype or outcome variable. We described this approach and the resampling methods used to ensure the robustness of association estimates [57,61].

3.3. Analytic considerations: systems biology

In bone biology, as in other domains, the primary methods used to determine signaling pathways have been reductionist: genes and proteins are altered one by one (e.g., using knock-out mouse models), and downstream effects are measured. These methods have provided the crucial basis for biology, therapeutics, and diagnostics. However, they cannot provide insight into the integrated and interacting actors within the skeletal system and between the skeleton and other systems. The various components of complex biological functions include genomics, transcriptomics, proteomics (proteins as well as various post-translational modifications) and metabolomics. Although omics are measured separately, they are inextricably linked biologically. Recent studies incorporating transcriptomic and proteomic data into genomic analyses have demonstrated substantial increases in the proportion of phenotypic variance explained and in the ability to pinpoint *causal* genetic variation [62]. Most of this work has been in animal models, which propel biologic understanding but do not represent human variation. Rapidly evolving integrative methods in computational biology and statistics can integrate these measures to more closely reflect the biological networks that influence phenotypic outcomes [63,64] and can substantially outperform single-omic approaches for predicting phenotypes [65,66]. A good example is the elucidation of the role of FTO in obesity [67]. There has been essentially no application of emerging integrative analytic techniques to musculoskeletal disorders in human population-based studies.

4. Proteomics in skeletal research

Despite the promise of new proteomic methods, studies using state-of-the-art proteomics in bone research are relatively limited; omic research in bone has been primarily focused on genomics and transcriptomics. Nevertheless, the application of newer omics measurements — including proteomics — to address questions of bone biology is increasing quickly, reflecting both the rapid evolution in proteomic technologies as well as the adoption of these approaches by the scientific community interested in bone.

4.1. Tissue-based proteomics

Some proteomic studies of bone have focused on in vitro systems. For instance, Baroncelli et al. [24] performed a comparative analysis of proteins from three osteoblast-derived extracellular matrix preparations that were osteopromotive for mesenchymal stem cells and that reflected different phenotypes with respect to mineralization. Using gel electrophoresis followed by mass spectrometry, they identified > 1000 proteins, including proteins that were uniquely present in mineralized vs unmineralized matrix including growth factors and proteins linked to cell migration and angiogenesis. Recently, Maretti et al. characterized skeletal mesenchymal stem cells [68] and Zhang et al. used proteomic methods to identify the proteins associated with exposure of mesenchymal stem cells to an antisense lncRNA that regulates osteogenesis [69]. The lncRNA altered expression of proteins involved in the actin cytoskeleton, focal adhesion, extracellular matrix-receptor interaction, and the spliceosome. Using human osteoblasts and endothelial cells, Simunovic et al. examined the proteome that was altered by co-culture of the two cell types, and confirmed their findings with immunosorbent assays [70]. Using LC-MS/MS-based proteomic analysis, Weirer et al. found evidence of osteoclast-specific proteins in atherosclerotic plaques [71], and other reports include proteomic evaluations of osteoblast, osteoclast and mesenchymal cell populations [18].

4.2. Blood-based proteomics

Proteomic methods are more often being applied to in vivo analyses. Clinically relevant skeletal outcomes evaluated with proteomic methods to discover and validate biomarkers include those that can be classified as leading to potential diagnostic (e.g., osteoporosis), predictive (e.g., fracture and bone loss), and prognostic (e.g., fracture healing) biomarkers. Each of these events is an ideal target for proteomic marker discovery, since lead time can be lengthy enough to allow for preventive intervention. In addition, proteomics of biofluids can provide valuable pathophysiological insights, especially when combined with tissue-level studies.

The plasma proteome is a collection of proteins from tissues and cells throughout the organism (including the skeleton) and is thus is an important mirror of biological events and a rich source of biomarkers [15,72]. On the other hand, the dynamic range of plasma protein concentrations is from mg/mL to ng/mL, or a factor of 10^{12}, making its description and study daunting [73,74]. Relatively few proteomic studies of skeletal outcomes have reported using plasma or serum. They include a study of recurrent fracture in children [75] and an evaluation of the proteins associated with BMD loss and hip fracture in older men. In the latter, we demonstrated some unique advantages of high-throughput proteomics. We used LC-MS/MS combined with ion mobility separation to examine the association of serum proteins with bone loss and fractures in participants (N = 2473) enrolled in the Osteoporotic Fractures in Men Study (MrOS) [57]. In these untargeted, discovery analyses, 20 proteins were associated with bone loss, and 5 of those were also associated with hip fracture. Bioinformatic analyses, including Gene Ontology (GO) term annotation, GO enrichment analysis, and analysis of protein-protein interactions revealed pathways that could be implicated. First, we were able to confirm several recently described markers of osteoporosis, like CD14 and SHBG. Novel associations were identified as well. Not surprisingly, proteins in inflammatory innate immune response pathways were associated with bone loss. Though these pathways are known to affect bone homeostasis, identifying protein markers readily detectable in serum draws this knowledge closer to clinical utility. More important for novel biomarker discovery, we were able to detect protein associations in pathways not already recognized for their role in bone. For example, the study pointed to proteins known to increase with aging and to co-occur with body composition changes.

Several other investigators have used derivations of blood samples,

including monocytes and serum-derived exosomes, arguing that the proteome of these subsamples is more biologically relevant to bone cell biology and cellular signaling than the broader circulating proteome. Indeed, characterizing the proteins in extracellular vesicles offers a unique opportunity to eavesdrop on the communication occurring within bone and with other systems, for example between bone and muscle [76–79]. Identification of biologically relevant proteins in these fractions can predict the processes of skeletal aging or facilitate disease diagnosis and prognosis. Zeng et al. [80] used liquid chromatograph-nano-electrospray ionization-mass spectrometry-based quantitative proteomic analysis combined with several bioinformatics methods (STRING (Search Tool for the Retrieval of Interacting Genes/Proteins), DAVID (Database for Annotation, Visualization and Integrated Discovery) and Cytoscape) to probe peripheral blood monocytes that can be precursors to osteoclasts in small groups of osteoporotic and control women. In a small comparison of 17 women with low hip BMD to 16 with high BMD, they found that concentrations of ITGA2B (integrin alpha 2b) were increased in the high BMD group, and that network analysis suggested that RhoA (Ras homolog gene family, member A; a small GTPase protein primarily involved with cytoskeletal regulation and signaling) was associated with the group differences. These findings were interpreted to implicate regulation of the actin cytoskeleton and leukocyte transendothelial migration in the genesis of osteoporosis risk. Others used similar approaches to study monocytes [58,59] or serum exosomes [23].

Large scale analyses using targeted proteomics, including plate-based ELISA proximity extension assays and aptamer-based protein assays, have also recently become feasible. With aptamer-based measures coupled with systems analyses, Emilsson et al. [9] examined 4137 proteins in 5457 older Icelanders to identify proteins and protein modules associated with cardiovascular and metabolic disease states. Hussein et al. [81] used similar methods, with expression analyses of fracture callous, to identify proteins and networks associated with fracture healing in mice. In a study performed in mice using SOMAmer assays, they probed > 1000 serum proteins over the time course of fracture healing and found that many varied during the stages of healing, including those apparently derived from bone (osteoblasts) and relevant signaling pathways (NF-κB, FGF, IL-6, Wnt/-βcatenin, BMP), as well as from other tissues (e.g. coagulation). There was considerable overlap between protein levels and RNA expression profiles in callus, but also some differences. They speculated that their findings might provide the basis for developing biomarkers for fracture healing. As described above, targeted proteomics in biofluids is also very feasible using measures based in mass spectrometry but has not been extensively utilized in skeletal research.

4.3. Summary

Proteomics is being used more often to better understand bone biology and related disease states. The synergistic use of multi-omic methods and systems biology is also emerging as a potentially powerful approach for understanding these issues. The path from protein association to a clinically useful biomarker or panel is known to be long [82]; however, casting a wide net through proteomic technology provides a singular opportunity for comparing novel markers to multiple other proteins simultaneously.

5. Conclusions and limitations

Deep quantitative proteomic analyses represent a compelling opportunity to better understand skeletal biology and disorders. Mass spectrometric (MS) and plate-based methods (using antibody-based and aptamer probes) that enable precise protein measurements are extremely attractive to define quantitative biomarkers of disease. Integrative multi-omic analysis approaches offer the opportunity to much more fully probe biological systems.

Though incredibly powerful, proteomic approaches are not without their limitations. Depending upon the MS-based global (discovery) analyses approach, we have previously discussed how sensitivity of quantification can be limited compared to other enrichment-based analyses (e.g., ELISA or aptamers). Mitigating strategies can improve peptide signal intensities and reduce detection missingness, but these involve increased analysis time with a trade-off of less overall throughput. High throughput MS methods are evolving quickly, but still can't match the throughput of plate-based targeted measurements. On the other hand, antibody and aptamer approaches do not provide the ability to detect and evaluate the richness of peptide sequence variation and post-translational modification. Targeted MS approaches in many ways provide advantages over the often variable specificity of antibody or aptamer style measures.

Acknowledgments

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