



Chapter 12

Myokines, Measurement, and Technical Considerations

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Abstract

Skeletal muscle has long been established as a highly multifunctional organ, playing a vital role in locomotion, whole-body metabolic and energy homeostasis, and thermoregulation. More recently, emergent evidence has highlighted a potent secretory role for muscle, producing and releasing “myokine” molecules that act in autocrine, paracrine, or endocrine fashion to govern muscle physiology and regulate whole-body homeostasis via multi-tissue cross talk mechanisms. Myokines represent promising therapeutic targets in health and disease, with their discovery, measurement, and functional importance being a hotbed of research across numerous physiological contexts. Here, we provide an overview of myokines and summarize current understanding of their biological role(s). We also outline primary approaches for myokine analysis, including detailed methodology for performing omics-driven myokine prediction, while further appraising both method-specific and general technical considerations to provide an evidence-based approach for designing and conducting myokine experiments.

Key words Skeletal muscle, Secretome, Myokine, Measurement, Analysis, Omics, Technical considerations

1 Introduction

Skeletal muscle represents the largest tissue constituent of the human body, comprising approximately half the total body mass of healthy individuals [1]. In occupying such a profound mass, skeletal muscle contributes significantly to multiple bodily functions essential for physical function and health [2]. The most notable is the muscles’ mechanical role, generating the force necessary to maintain posture and enable movement [2]. Muscle tissue also facilitates thermoregulation and respiration [3, 4] and, as the body’s largest substrate (protein, fat, and carbohydrate) store, plays a vital role in whole-body energy metabolism [2]. Beyond these well-established functions, however, recent evidence points toward skeletal muscle as a prominent secretory organ, responsible for the production and release of small molecules termed “myokines” that perform autocrine/paracrine roles to directly influence the form and function of muscle and/or endocrine roles to facilitate multi-

tissue cross talk and regulate whole-body physiology [5–8]. Myokines are sensitive to a variety of different (patho)physiological stimuli, including nutrients, stress, hormonal alterations, environmental factors, metabolic dysfunction, and aging [9–11]. Also a potent stimulator of myokine expression/secretion is muscle contraction itself [6, 7, 12], with myokines holding promise as biomarkers for positive exercise-related health adaptations, as well as pharmacological “exercise mimetic” targets in clinical populations [8, 10, 13–15].

Accurate and robust myokine analyses are essential to avoid critical errors in detection and interpretation that would hinder clinical myokine progress [8]. Numerous technical constraints must, therefore, be considered when measuring and quantifying myokines in a biological sample, from pre-analytical sample collection and handling to the specific choice of downstream analytical approach(es). As such, this chapter aims to provide a technical road map of robust myokine identification and physiological experimentation by overviewing myokines and their known biological roles; outlining and technically critiquing existing approaches for myokine analysis; emphasizing the utility of contemporary informatic methodologies; and appraising wider technical and experimental considerations for designing and conducting myokine experiments.

2 Myokines: Characterization and Biological Function(s)

2.1 Defining Myokines

At the turn of the twenty-first century, Bente Klarlund Pedersen and colleagues demonstrated that working skeletal muscles produce and systemically release the now well-known humoral factor (“cytokine”) interleukin (IL) 6 (IL-6) [16]. The term “myokine” was subsequently coined to describe cytokines (a broad class of small signaling molecules typically with masses <30 kDa) produced and released by skeletal muscle, which exert effects in other parts of the body [17]. Nevertheless, other myokine molecule types have since been described, and more recent myokine classifications typically extend to include both cytokines and other non-cytokine proteins [5, 8, 18]. Myokines were also proposed as myofiber-derived factors [19], but, with muscle being a complex heterogeneous tissue [20], the term is frequently used to describe factors produced and released by any muscle subcompartment/organelle, which together encompass the wider muscle “secretome” [5]. Moreover, though myokines have become synonymous with factors produced and released in response to contraction, they can also be regulated by other (patho)physiological stimuli and/or constitutively secreted [9–11].

Contrary to earlier descriptions of myokines as endocrine factors [17], recent evidence suggests that myokines may primarily act as local agents to protect and improve the functionality of muscle

by governing intramuscular cross talk between myofibers, immune cells, fibroblasts, vasculature, and bone [5]. Numerous molecules defined as myokines can also be secreted by tissues other than muscle, such that muscle exclusivity cannot be presumed from systemic myokine identification [21]. Nonetheless, given muscles' substantial contribution to total body mass, skeletal muscle is likely a primary, if not the only, source of many secreted factors observed in circulation [10], particularly in response to large-scale muscular work such as whole-body exercise. Lastly, growing evidence indicates that muscle secretory factors include not only protein or peptide molecules but also other regulatory molecule classes such as metabolites/lipids ("myometabokines") and noncoding micro-RNAs ("myomiRs") [5, 9, 22], a detailed discussion of which is considered beyond the scope of this chapter. All things considered, this chapter employs a broad definition of the term "myokine" to include any secreted protein or peptide that is capable of being produced in skeletal muscle, regardless of stimulus and whether they act in an autocrine, paracrine, or endocrine fashion [5].

2.2 Biological Functions of Myokines

Myokines have been implicated in diverse biological processes, which have been extensively reviewed elsewhere [5, 8, 18, 21, 23–25]. A dominant feature of myokines described thus far is direct intramuscular regulation, influencing skeletal muscle physiology via diverse mechanism(s) [13, 18, 25]. For instance, myostatin (or growth differentiation factor 8; GDF-8), one of the earliest identified myokines [26], governs myogenic and anabolic signaling-mediated control of muscle mass [27]. Numerous other myokines also putatively act in an autocrine/paracrine fashion to regulate myogenesis and/or muscle trophic-related signaling (e.g., IL-4, IL-6, IL-7, IL-15, decorin, insulin-like growth factor 1 (IGF-1), musclin, and leukemia inhibitory factor (LIF)). Myokines also exert important regulation over muscle metabolism (e.g., IL-6, IL-13, IL-15, brain-derived neurotrophic factor (BDNF), and angiopoietin-like 4 (ANGPTL4)) and mitochondrial biogenesis (e.g., musclin, myonectin, and fibroblast growth factor 21 (FGF-21)). Remodeling of the vasculature (e.g., IL-8, vascular endothelial growth factor (VEGF), and cysteine-rich angiogenic inducer 61 (CYR61)) and extracellular matrix (e.g., secreted protein acidic and cysteine rich (SPARC) and follistatin-like 1 (FSTL1)) is also influenced by myokines, as are immune/inflammatory responses (e.g., IL-10, c-c motif chemokine ligand 2 (CCL2), and fractalkine) [5, 8, 12, 13, 18, 23, 25].

The complexity of myokine function is further illustrated by their capacity to communicate with a variety of distal tissues. For example, the dominant metabolic effects of muscle-derived IL-6 (at least after exercise) are suggested to extend to adipose tissue and the liver [28, 29]; IGF-1 is implicated in bone formation [21]; and IL-15 might influence skin aging [8]. However, since the majority

of myokines might work primarily in an autocrine/paracrine manner, future research might focus on the muscle-intrinsic effects of novel myokines. For example, muscle-derived BDNF acts locally to mediate muscle fat oxidation, satellite cell differentiation, and muscle regeneration but is unlikely an endocrine factor [8, 23, 25], with secretion from the brain as the major source of circulating BDNF [30, 31]. Nonetheless, despite much progress, biological function is said to have been established for only a small portion (~5%) of identified myokines [8]. Contributing to this incomplete understanding are technical difficulties when conducting myokine experiments and analyses. This chapter will, therefore, discuss key methodological approaches and associated technical considerations that will facilitate the identification of novel myokines. To assist subsequent exploration of myokine roles in regulating muscle and whole-body physiology, we further highlight important aspects of analytical methods to consider when to design and conduct myokine-centric experiments.

3 Primary Approaches Toward Myokine Analyses

This section outlines primary approaches for performing myokine analyses and critical method-specific considerations. While not exhaustive, methods covered focus on those most commonly employed to measure myokines in vivo (i.e., skeletal muscle tissue and blood samples) and in vitro. Further, most of these techniques are not myokine-specific analytical tools but rather are routine wet lab techniques used more generally in molecular research. Thus, while we do highlight method-dependent considerations of importance to myokine detection/quantification, a number of relevant methodological aspects are generalized, myokine-independent technical constraints and will not be discussed in great detail.

3.1 Primary Techniques for Targeted Myokine Analysis

By far the most common form of myokine detection/quantification is hypothesis-driven analysis of one or a finite number of target myokines. Well-established methods for performing targeted myokine analysis center upon polymerase chain reaction (PCR), immunoassays, and immunohistochemistry (IHC).

3.1.1 Real-Time PCR (qPCR)

The qPCR method is used to measure mRNA abundance in a biological sample, permitting myokine detection/quantification at the transcriptional level. Basic principles of this method are reviewed in [32], and a wide range of commercial kits are available from multiple manufacturers for performing both single and multi-molecule qPCR analysis of target myokines and associated functional molecular classes, e.g., the TaqMan™ Array Human Cytokine Network (Cat No. 4418769, Thermo Fisher Scientific), which permits the simultaneous measurement of mRNA abundance for

28 cytokine molecules. qPCR subsequently offers a fast, robust, cost-effective, and relatively high-throughput method for hypothesis-driven myokine measurement, at the same time providing a high degree of sensitivity while remaining sample-efficient [33, 34].

A high degree of sensitivity is particularly advantageous, with many myokines having a low abundance, especially in the healthy basal state [35]. However, this low abundance of many myokines renders some general aspects of robust PCR even more relevant when examining myokine changes. For example, while it is theoretically possible to measure mRNA abundance in absolute terms using this method, greater accuracy is attained when measuring relative differences between conditions [36] (e.g., baseline versus postexercise). Additionally, to further ensure accurate results, mRNA abundance should be normalized to a reference gene that is unperturbed across conditions, to control for differences in starting RNA concentrations and reaction efficiency [36]. However, care should be taken to ensure that chosen housekeeping genes are appropriately stable across conditions, for example with muscle contraction, where commonly used reference genes such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) can be robustly increased by acute exercise [37]. Such reference genes should, therefore, be used with caution due to the potential for masking biologically meaningful expression changes in low-abundance myokines. Overall, PCR offers a gold standard in sensitive quantitative assessment of myokine transcript changes. When appropriately executed and combined with key experimental considerations (*see* Subheading 4), PCR can robustly determine myokine responsiveness to external stimuli and validate gene-level expression of novel candidate myokines identified through untargeted techniques (*see* Subheading 3.2). Accordingly, making sure that the primer aligns to sequences used to map myokines from transcriptomics is an important consideration. Moreover, in developing a multi-myokine PCR assay, one should ensure that all primer-probe sets are compatible with one another in accord with the exact set of myokine targets chosen for analysis [36].

3.1.2 Immunoassays

Immunoassays permit myokine detection/quantification at the protein level and allow measurement of a limited number of target proteins. Immunoassays regularly used for myokine research are the enzyme-linked immunosorbent assay (ELISA), Western blotting, and antibody arrays. Each method associates with certain unique methodological constraints that should be considered when applied to myokine-centered research, briefly outlined below. However, all immunoassay approaches converge on a common principle: quantifying antigen (herein myokine) content via the magnitude of antigen binding with a specific corresponding antibody [38]. Ensuring excellent antibody specificity is thus the single most important

technical item across immunoplatfroms for robust myokine analysis, failure of which is a frequent source of detection error. A stark reminder of the need for robust antibodies is the recent controversy surrounding the biological relevance of irisin upon its discovery as a myokine, with measures of serum irisin later identified as an artifact of poor ELISA specificity [39]. Thus, appropriate scientific rigor in antibody design and, crucially, proper antibody validation prior to experimentation are essential to avoid significant wasted myokine-related research efforts.

ELISA

First described half a century ago [40], the ELISA technique remains one of the most utilized methods for targeted myokine analysis, particularly in biological fluid samples (blood and cell culture supernatant). Well-validated, commercially available ELISA kits exist for many (candidate) myokines. For example, Invitrogen™ currently offers individual ELISA kits for a broad menu of over 700 cytokine, chemokine, and growth factor targets (thermofisher.com). ELISAs are time-efficient per assay, highly sensitive, and quantitative [41, 42]; however, serial sample dilutions are often required to account for the often narrow dynamic range of many ELISAs, potentially exaggerating differences between samples falling inside versus outside the dynamic range [43]. This factor might be particularly influential when analyzing myokines, since frequently low basal levels may, in some instances, increase up to 1000-fold upon demand [44]. Standard ELISAs also only permit measurement of one analyte at a time, and so, large sample volumes, time commitment, and financial cost would be required to analyze multiple myokines using this approach in isolation [42].

Western Blot

Western blotting (immunoblotting) is a cornerstone of modern physiology research. While less sensitive than ELISAs, Western blotting can afford greater target specificity by virtue of electrophoretic separation of proteins within a sample by their molecular weight and can thus be applied to delineate inactive and active protein forms, as well as different splice variants [33]. Both methodological features are advantageous for myokine analysis, since more than two-thirds of genes encoding secreted proteins have at least one splice variant with different cellular location (s) [14, 45]. Myokine splice variants thus possibly possess divergent biological roles, and, indeed, the biological potency of certain target myokines (e.g., IL-1 β) can be distinguishable by molecular weight [33]. Other general technical considerations associated with Western blotting equally apply to myokine studies (comprehensively reviewed by Bass and colleagues [46]). However, specific to myokine analysis, the relative validity of using a non-secreted versus secreted reference/loading control protein in a given context is

worth considering (e.g., the validity of using a non-secreted protein as a reference/loading control for Western blotting of biological fluids) [47]. As an alternative that circumvents the need to identify (non)secreted reference proteins, Coomassie staining represents a reliable approach for standardizing total protein load [46].

A feature of many myokines is low molecular weight [19, 48]; thus, more specific considerations for Western blot can include appropriate gel concentration (20% recommended for proteins <20 kDa) and running buffer (2-(N-morpholino)ethanesulfonic acid (MES) suggested for proteins <36 kDa) choices during electrophoresis and membrane pore size (smaller advised for proteins <20 kDa) selection during protein transfer [46]. Unlike ELISAs, Western blotting permits measurement of multiple myokines in a single analysis via membrane strip and reprobing (up to three times being recommended) and/or membrane dissection by molecular weight (assuming appropriate prior antibody validation) [46]. However, as well as ensuring antibody specificity for each analyzed target, extreme care should be taken when choosing myokine target combinations given how close myokines can be across low molecular weight ranges [49].

Antibody Arrays

Antibody arrays are an extension of traditional ELISA methods, which allow simultaneous measurement of multiple myokines in the same sample [42]. These arrays typically exist in one of two forms: planar or bead-based. In planar arrays, individual antibodies are immobilized in microarray format and probed with the sample of interest. Conversely, bead arrays utilize color-coded and size-coded microbeads to permit protein-specific binding and identification and are generally most commonly employed [33]. Exploiting microbeads suspended in a sample versus fixed to a platform offers superior binding kinetics, limits of detection, and broader dynamic detection ranges than traditional ELISAs [42, 50], minimizing serial sample dilutions. However, broad/varying dynamic ranges can be problematic when assaying low- and high-abundance targets in tandem [43]. Sensitivity/comparability with conventional ELISAs may also vary across individual myokines and/or biological samples (e.g., tissue culture supernatant versus plasma samples) [43, 51]. When customizing myokine combinations on a single array, it is further critical to consider non-reactivity of individual targets with all other antibodies, and a reliable uniplex assay is not necessarily suitable for addition to a functioning multiplex assay [42]. After mitigating these factors, antibody arrays can be customized for desired myokine targets, with several arrays focused on specific myokines or functional classes of myokine also commercially available (e.g., the MILLIPLEX[®] MAP Human Myokine Magnetic Bead Panel containing 15 established myokines (Cat No. HMYOMAG-56K, Merck Millipore) or equivalent cytokine/

chemokine panels that contain upward of 40 targets (Merck Millipore)). As such, antibody arrays can offer substantially higher throughput, accurate myokine analysis versus traditional ELISAs and Western blotting while being cost-, time-, and sample-efficient [33].

3.1.3 *Immuno-histochemistry (IHC)*

IHC is categorized as an immunoassay technique since protein detection is achieved using antigen-antibody binding and is thus subject to many of the above technical caveats. However, IHC also determines target protein location in intact muscle tissue sections [52], which is important given the tight link that exists between protein spatial localization and function [53]. Most IHC technical considerations are generic to biological studies [54], and with appropriate primary/secondary antibody combinations [36], colocalization of multiple target myokines can be assessed in a single tissue section. Like Western blotting, selecting antibodies in accord with biological potency of interest is particularly relevant to myokine analysis, since some (candidate) myokine targets can be synthesized in a specific form (e.g., IL-1 β , which can be synthesized in inactive form) [36], with possible functional consequences.

When employed across multiple physiological time points, IHC further complements myokine studies with temporospatial information to infer functionality beyond expression changes alone (e.g., subcellular site of action and colocalization-based inferences of multi-myokine functional similarities/differences). Classical protein secretion involves sequential movement from the cytosol to secretory vesicles via the rough endoplasmic reticulum lumen and Golgi cisternae [55, 56] and is thus presumably the main route of secretion for myokines that contain a signaling sequence. Conversely, myokines (e.g., fibroblast growth factor 2; FGF-2) can lack a signaling sequence but be secreted through alternate means such as via small exosome vesicles [8, 45, 57, 58]. By assessing temporal colocalization of myokines with specific subcellular markers (e.g., CD63—an exosomal marker protein), the temporospatial capacity of IHC could thus also serve as a useful tool to help identify myokine mechanisms of secretion [59–61]. A further such advantage [36] is the ability to assess myokine production by cell type. For example, contraction-induced muscle production of IL-6 occurs preferentially in type 2 versus type 1 myofibers in vivo [62]. Combined, given the capacity for dynamic, location- and cell-specific myokine function, significant new functional information can be gained by including IHC analyses in myokine research.

3.2 *Approaches Toward Untargeted Myokine Discovery*

Rapid technological advancements have paved the way for a shift toward a new era of modern molecular analyses, termed the “omics” revolution [63, 64]. Omics-based tools make it feasible to simultaneously characterize the molecular state of a sampled cell/tissue at an extremely high resolution. This feature facilitates

“untargeted” molecular study and, in turn, provides a powerful platform to generate new hypotheses and identify novel molecular signatures and regulatory control mechanisms underpinning phenotypic change [63, 65–67]. Accordingly, omics applications have emerged as prime tools toward discovering new myokine candidates for subsequent hypothesis-driven work [45]. Main omics tracts for unbiased myokine analyses are transcriptomics and proteomics: the global study of mRNA and protein levels in a sample, respectively [66]. Transcriptomic data are typically generated by means of microarray or RNA-sequencing (RNA-seq). While microarrays are cost-effective and can enable the expression of tens of thousands of genes to be quantified simultaneously, RNA-seq affords significantly higher resolution and lower limit of detection, allowing quantification of previously annotated genes plus novel features and, in turn, theoretically permitting measurement of all mRNA species in a cell/tissue [68–72]. Proteomic data are normally attained using mass spectrometry, either via a top-down or bottom-up strategy. Top-down methods directly analyze intact proteins, whereas bottom-up approaches analyze proteins on the peptide level; the latter are perhaps preferable for studying myokines, whereby low abundance can be problematic due to greater sensitivity, larger dynamic range of detection independent of protein size, isoelectric point or hydrophobicity, and a greater degree of automation [22, 24]. The relative advantages/disadvantages of transcriptomics and proteomics for untargeted myokine discovery are further discussed in Subheading 5.

An extensive description on conventional transcriptomic/proteomic data analysis is beyond the scope of this work, for which the reader is directed to several excellent online resources [73–78]. Transcriptomic/proteomic experiments typically yield a list of genes/proteins that are differentially regulated across two or more sets of samples and/or central, highly connected (“hub”) molecules in the context of a large-scale molecular interaction network. Thereafter, prediction of myokine candidates can be achieved by unbiasedly screening differentially regulated/hub molecule lists for possible secreted factors—a process termed “secretome” analysis. A number of predictive bioinformatic tools then exist that can facilitate untargeted myokine discovery. A notable mention is SignalP, an online bioinformatic tool that uses sequence alignments and machine learning-based algorithms to predict secreted proteins [22, 79]. Curated knowledge bases can also serve as useful for identifying secreted factors. These include the Gene Ontology (GO), the world’s largest source of information on gene function that is organized into logical classes [80]. For myokine identification, putative secreted molecules can be filtered under extracellular-related cellular component classes (e.g., “extracellular region” and “extracellular space”) [15]. Additional

knowledge bases include the Universal Protein Resource (UniProt) database [81], which contains reviewed and computationally predicted secreted molecule lists (under UniProt keyword “Secreted”).

In SignalP, secreted proteins are predicted based on the presence of an N-terminal cleavable signal sequence, and thus, such an approach is effective for elucidating myokine candidates secreted via classical means. However, as already noted, many secreted factors including myokines do not carry a signal sequence and are, therefore, likely secreted through nonclassical means, such as via exosomes [8, 45, 57, 58]. In which case, specific bioinformatic tools for sequence-based prediction of nonclassically secreted proteins are further available [82], such as the SecretomeP web server [83], as well as other tools more aimed at specifically predicting protein secretion by exosomes (e.g., ExoPred [84]). Many proteins secreted into the endoplasmic reticulum are also sorted to various intracellular compartments (mitochondria, lysosomes, etc.) or retained in the endoplasmic reticulum/Golgi cisternae, rather than being secreted from the cell [85]. Further filtering by subcellular localization may thus also be informative, which can be aided by several predictive tools including DeepLoc [86] and TargetP [87]. False-positive prediction of classical or unconventional secretion can further arise from proteins containing transmembrane domain [82, 88]. Accordingly, updated iterations of SignalP include algorithms that aim to better discriminate signal peptides from transmembrane regions with higher precision (e.g., [89]). Additional stand-alone tools that directly predict transmembrane helices in protein sequences can also be integrated to eliminate transmembrane molecules from predicted secretory factor lists, such as the transmembrane hidden Markov model (TMHMM) web server [90]. A recommended [82] and common [15, 35, 91, 92] approach is, therefore, to integrate multiple bioinformatic tools when screening for putative secretory factors. Further screening of myokine candidates for preferential skeletal muscle expression may help add an extra layer of confidence in muscle as the predominant origin of production/release [15]. Experimentally tractable sets of novel candidate myokines, responsive to particular (patho)-physiological stimuli, can henceforth be discovered for subsequent hypothesis-driven work and validation as definitive myokines.

With omics approaches now well at the forefront of modern molecular discovery, the current chapter focuses on providing extended methodological detail for performing omics-driven myokine prediction, found in the next section. Notably, we outline a handful of protein sequence-based prediction tools that can be incorporated into untargeted myokine discovery pipelines. It is important to highlight, however, that the selection of tools given is by no means exhaustive, with those presented chosen to give the reader a flavor of both utilizing and integrating sequence-based

prediction tools of differing purposes toward a workflow for untargeted myokine prediction. Nevertheless, even tools that hold a similar purpose (e.g., identifying proteins with a signal sequence) can vary in the underlying algorithm and may therefore generate marginally different results [14, 85]. As such, we also suggest a viable alternative/complementary tool that could help contribute to “standardized” myokine predictive screening (at least when analyzing human samples).

4 Methodology for Omics-Driven Myokine Prediction

4.1 Protein Sequence-Based Prediction of Candidate Myokine Targets

4.1.1 Obtaining Protein Sequences for Input into Prediction Tools

Given a (typically long) list of molecules derived via omics analyses (differentially expressed genes/proteins, network “hub” genes/proteins, etc.) of in vivo (muscle tissue and/or blood) or in vitro (muscle cell line and/or supernatant) samples, a required first step toward filtering for potential myokines is obtaining associated protein sequences for input into sequence-based prediction tools. Various knowledge bases exist that provide such detail across a variety of different organisms. A precise choice could simply be a matter of user preference and/or driven by other pertinent factors, such as methodological specificities. For example, if genome build and annotation files for RNA-seq analysis were used from a particular database, obtaining associated protein sequences directly from the same source may provide greater consistency and/or optimize database coverage (exhaustive discussion on all pertinent factors is considered beyond the scope of this work). With the predominant focus of this section being on the screening application per se, we therefore restrict focus on describing how to obtain associated protein sequences for gene/protein lists using two popular databases: Ensembl [93] and UniProt [81]. It is worthwhile noting that both tools provide associated sequences at the isoform level. If omics data have been summarized on a per gene/protein basis (rather than on an individual isoform basis), the user may thus wish to either focus on a specific isoform of interest or analyze all isoform-specific sequences per gene/protein molecule and then define a molecule as a myokine candidate if at least one variant pertains to the required criteria. The latter is perhaps more straightforward from a screening viewpoint, though the possibility of myokine splice variants having divergent biological roles (or even regulation and/or localization) may remain a potential caveat.

Ensembl

The BioMart tool within Ensembl (available through the Ensembl homepage: <https://useast.ensembl.org/index.html>) can be used to obtain protein sequences for multiple targets of interest in batch. Initially, one is required to choose a database option from the “Dataset” field (e.g., “Ensembl Genes” → “Human genes”). The “Filter” field can then be used to restrict the query to only those

targets of interest (e.g., “GENE:” → “Input external references ID list [Max 500 advised]” → “Gene Stable ID(s) [e.g. ENSG00000000003]” then paste or upload list of corresponding ID’s), and the “Attributes” field is used to specify inclusion of associated peptide sequences in the output (select “Sequences” then “SEQUENCES:” → “Peptide”). Peptide sequences per transcript per gene can consequently be downloaded in FASTA format (“Results” → “Export all results to” → “File” → “FASTA” → “Go”), a common text-based file format for representing protein sequences. Such a tool can accept several different types of gene and protein identifier as input.

UniProt

UniProt supports batch protein sequence retrieval via its “Retrieve/ID mapping” function (accessible via the UniProt homepage: <https://www.uniprot.org>), with pasted or uploaded lists able to be submitted upon “From” (target list ID type, e.g., “UniProt Gene name,” etc.), “To” (specific UniProt database to map to, e.g., “UniProtKB”), and “Organism” (e.g., “Homo sapiens (Human)”) option selections. Consequent results can be further filtered (“Filter by” parameter) to include manually annotated and reviewed (Swiss-Prot) and/or automatically annotated but unreviewed (TrEMBL) database records with the “Columns” option enabling for protein sequences to be appended to the results table (“Columns” → check “Sequences” under the “Sequences” subfield of “Add more columns”). UniProt-defined canonical only or canonical plus other isoform protein sequences for inputted targets can then be readily downloaded in FASTA file format (“Download” → “Download all” → “File format” either “FASTA (canonical)” or “FASTA (canonical and isoform)” → choose “Compressed” or “Uncompressed” file format → “Go”). Like Ensembl, such a tool supports a variety of different gene and protein ID inputs.

4.1.2 Signal Peptide Prediction via SignalP

As noted, classically secreted proteins carry a signal peptide with SignalP, a popular sequence scanning tool for establishing the presence and cleavage site location of possible signal peptides in proteins. A recent iteration of SignalP at time of writing is SignalP 5.0 (accessible at <http://www.cbs.dtu.dk/services/SignalP/>) [94], which can distinguish between “standard” secretory signal peptides (Sec/SPI), “lipoprotein” signal peptides (Sec/SPII), and “Tat” signal peptides (Tat/SPI). Desired protein sequences can be either pasted or uploaded in FASTA format with further options to define “Organism group” (“Eukarya” (default), “Gram-positive” (bacteria), “Gram-negative” (bacteria), or “Archaea”—n.b.: discrimination between the given three types of signal peptide is only possible for bacteria and archaea) and “Output format” (i.e., whether (“Long format”) or not (“Short format”) to include figures illustrating predicted cleavage site location) prior to submission (n.b.:

input protein sequences should be no less than 10 and no longer than 10,000 amino acids, with the maximum number of input sequences being 5000). A summary text file that includes the type of prediction (Sec/SPI, Sec/SPII, Tat/SPI, or OTHER if not predicted to contain a signal sequence) and location of cleavage site (if any) for all uploaded protein sequences can then be readily downloaded (“Downloads” → “Prediction summary”). Additional developer guidance for running SignalP 5.0 can be found at <http://www.cbs.dtu.dk/services/SignalP/instructions.php>.

4.1.3 Prediction of Nonclassically Secreted Proteins Using SecretomeP

To further probe for nonclassically secreted proteins (i.e., secreted factors that do not carry a signal sequence) by means of sequence-based prediction, SecretomeP represents a popular tool [83], the most recent iteration at time of writing being SecretomeP 2.0 (<http://www.cbs.dtu.dk/services/SecretomeP/>). Again, protein sequences can be either pasted or uploaded for submission in FASTA format (restricted to 100 sequences and 200,000 amino acids per submission, with each sequence being no less than 40 and no more than 4000 amino acids) and with further option to define organism prior to submission (specific options being “Gram-negative bacteria,” “Gram-positive bacteria,” and “Mammalian”). The provided results per sequence include an “NN-score” and “Warning.” As described in the results output, nonclassically secreted proteins should have an NN-score (“SecP score”) above a threshold of 0.5 for bacterial sequences and 0.6 for mammalian sequences and not flag a warning of being predicted to contain a signal peptide. Result outputs can be copy/pasted and stored in text file format for subsequent reference. Additional developer guidance for using SecretomeP 2.0 can be found at <http://www.cbs.dtu.dk/services/SecretomeP/instructions.php>.

4.1.4 Prediction of Protein Subcellular Localization Using DeepLoc

As mentioned, not all proteins secreted into the endoplasmic reticulum necessarily end up being secreted from the cell. DeepLoc [86] consequently presents as a protein sequence scanning tool for predicting subcellular localization, integration of which can thus add extra confidence that a protein may indeed be secreted. The most recent version at time of writing is DeepLoc 1.0 (available at <http://www.cbs.dtu.dk/services/DeepLoc/>), which can discriminate between ten different localizations: cell membrane, chloroplast, cytoplasm, endoplasmic reticulum, extracellular, Golgi apparatus, lysosome/vacuole, mitochondrion, nucleus, and peroxisome. Pasted or uploaded FASTA format protein sequences (not less than 10 or more than 6000 amino acids in length) can be submitted for scanning with an additional option (“Protein encoding”) to define preference for either accurate (“Profiles (accurate, 50 sequences maximum)”) or fast (“BLOSUM62 (fast, 500 sequences maximum)”) processing. Generated results include

an overall prediction of subcellular localization as well as whether a given protein is soluble or membrane with resultant data for all inputted sequences available to download as a single .csv file (“Downloads” → “CSV summary”). From a secretome analysis standpoint, extracellular localization presents as a logical filtering choice to include. Further guidance provided by the developers of DeepLoc 1.0 can be found at <http://www.cbs.dtu.dk/services/DeepLoc/instructions.php>.

4.1.5 Prediction of Transmembrane Helices in Proteins Using TMHMM

As elucidated, transmembrane proteins can exaggerate false-positive secretion hits, and additional confidence may thus be gained by incorporating stand-alone sequence-based transmembrane region prediction tools. A prominent option is TMHMM [90]; the most recent version (v2.0) of which can currently be located at <http://www.cbs.dtu.dk/services/TMHMM/>. As above, protein sequences can be either pasted or uploaded in FASTA format with restrictions being a maximum of 10,000 sequences and 4,000,000 amino acids per submission plus each sequence no more than 8000 amino acids. Prior to submission, there is also an option to define “Output format” (“Extensive, with graphics,” “Extensive, no graphics,” or “One line per protein”), as well as “Other options” (viz. whether to use old model—i.e., TMHMM v1). The “One line per protein” output includes per input protein, the length of protein sequence, expected number of amino acids in transmembrane helices, expected number of amino acids in transmembrane helices in the first 60 amino acids, number of predicted transmembrane helices, and associated predicted topology. Per input protein, extensive results further include a statistic on the total probability that the N-terminal is on the cytoplasmic side of the membrane, a warning if a predicted transmembrane helix in the N-terminal could in fact be a signal peptide (occurs when the expected number of amino acids in transmembrane helices in the first 60 amino acids of a given protein exceeds 10) and a list (if any) of locations of predicted membrane helices and intervening loop regions. In which case, if the whole sequence is labeled as inside or outside, the concomitant prediction is that the given protein contains no transmembrane helices (and thus recommended not to interpret as a prediction of location). If the expected number of amino acids in transmembrane helices exceeds 18, then it is said likely to be a transmembrane protein (or have a signal peptide). The “Extensive, with graphics” option further provides plots showing the posterior probabilities of inside/outside/transmembrane helix. Result outputs of the “One line per protein” option are highly suitable for being copy/pasted and stored in text file format for subsequent reference. Full guidance provided by the developer can be found at <http://www.cbs.dtu.dk/services/TMHMM/TMHMM2.0b.guide.php>.

4.1.6 Prediction of Exosome-Secreted Proteins Using ExoPred

Many tools for predicting nonclassically secreted proteins, including SecretomeP, incorporate algorithms that were not trained toward the specific intent of predicting protein secretion by exosomes and thus may not perform optimally in doing so [84]. Moreover, some myokine candidates that neither contain a signal sequence nor display extracellular localization may still yet be secreted by means of exosome transportation [45]. In which case, a recent complementary sequence-based tool for predicting myokines unconventionally secreted via exosomes is ExoPred [84], currently available at <http://imath.med.ucm.es/exopred/>. Such a tool too accepts either pasted or uploaded protein sequences in FASTA format with additional options to include subcellular location as defined by UniProt and/or predicted by Psort (another protein subcellular localization prediction tool [95]) in the results. Clicking run will consequently generate the results table, wherein the “ExoPred” column details whether (“Y”; yes) or not (“N”; no) input proteins can be secreted by exosomes (n.b.: as part of its processing, “ExoPred” also detects proteins with signal sequences (via SignalP) and/or transmembrane regions (via TMHMM), of which are consequently assigned a “NA” (not available) prediction in the “ExoPred” column). Result outputs can be copy/pasted and stored in text file format for subsequent reference. Further developer guidance for using ExoPred can be found at <http://imath.med.ucm.es/exopred/instructions.html#UG>.

Given the abovementioned tools, a logical workflow for their integration within omics-driven myokine prediction is outlined in Fig. 1 (though input could quite easily be associated protein sequences of any viable gene/protein list of interest). As noted, however, a variety of other pertinent sequence-based tools exist with aims adjunct to the specific tools described above, and distinct tools with a similar purpose may not necessarily provide identical results. For example, SignalP 4.0, Phobius, and SPOCTOPUS signal peptide prediction tools have shown to yield a differing number of hits to one another upon whole-proteome analysis [85]. Thus, while the workflow per se presented in Fig. 1 may be viable for candidate myokine screening, a more “standardized” approach could be to integrate several different sequence-based tools and even knowledge base annotations (e.g., GO cellular component terms for protein localization filtering) at each stage. In which case, a viable option that may help contribute to standardized candidate myokine screens (at least in humans) is the Human Protein Atlas-defined human secretome [14, 85].

4.2 Putative Myokine Screening via the Human Protein Atlas

4.2.1 Human Protein Atlas-Defined Human Secretome

The Human Protein Atlas-defined human secretome is a readily available dataset containing 1708 genes conservatively predicted to have at least one secreted protein variant as based on whole-proteome analysis of all Ensembl transcripts using a compendium of bioinformatic tools, including a vast array of different sequence-based prediction approaches. Notably, this list comprises genes

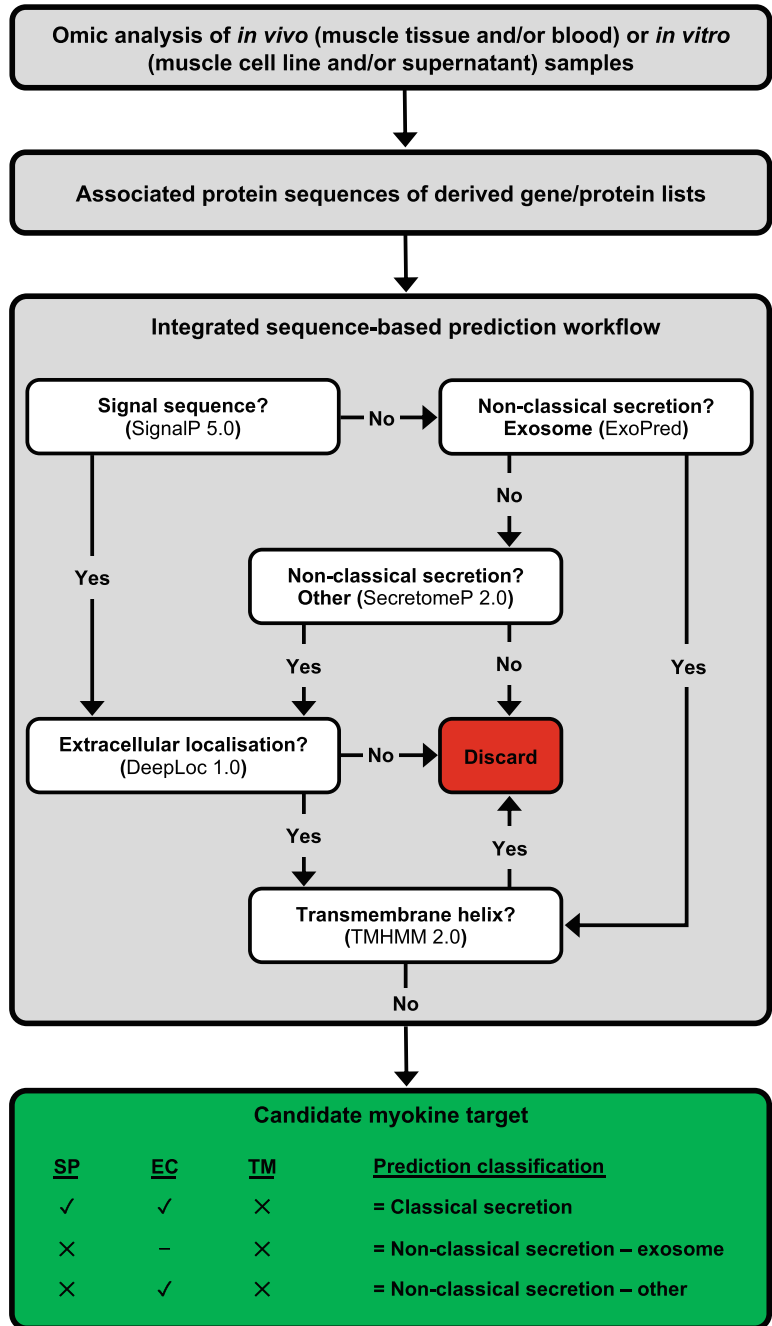


Fig. 1 Omics-driven myokine prediction workflow. Central flowchart highlights example workflow for screening candidate myokines by means of protein sequence-based prediction tools. As noted in-text, since ExoPred itself omits proteins with transmembrane regions, positive hits obtained using this tool should naturally pass the subsequent TMHMM transmembrane helix screening stage. Abbreviations: EC extracellular, SP signal peptide, TM transmembrane

having at least one protein variant: (i) predicted to contain a signal peptide based on a majority decision method of three sequence-based tools (SignalP 4.0, Phobius, and SPOCTOPUS) with no predicted transmembrane region based on majority decision method of seven sequence-based tools (MEMSAT3, MEMSAT-SVM, Phobius, SCAMPI, SPOCTOPUS, THUMBUP, and TMHMM) or otherwise annotated under the UniProt keyword “Secreted” and (ii) not annotated as intracellular and/or membrane-bound (e.g., endoplasmic reticulum or Golgi residing, mitochondrial, lysosomal, membrane-associated, etc.). Such a tool therefore substantially minimizes the need for the user to directly employ multiple prediction tools and may thus present as one possible “to hand” source for helping standardize large-scale myokine predictions across independent studies. It is worthwhile noting however that, since the Human Protein Atlas-derived secretome largely stems from predictions made on signal peptide presence, unconventionally secreted proteins may not be fully encompassed, and it may therefore be most suitable as a standard for screening of classically secreted myokines. In which case, an updated iteration or stand-alone process that incorporates multi-method prediction of unconventionally secreted proteins (e.g., SecretomeP, SRTpred, SecretP, SPRED, OutCyte, ExoPred, etc. [84, 96]) may be desired as an alternative/complementary standard.

The complete list of molecules that comprise the Human Protein Atlas-defined human secretome can be found at <https://www.proteinatlas.org/humanproteome/tissue/secretome>, by clicking on “Predicted secreted proteins” within Table 2. The resultant data table can then be downloaded as a .tsv file in its entirety (i.e., including columns for all pertinent Human Protein Atlas database annotations; click “.TSV” option) or with custom data columns (click “Custom TSV/JSON” then “Download TSV”; default data columns included this way, along with Ensembl Gene ID’s, are “Gene,” “Gene description,” and “Evidence”).

4.2.2 Using the Human Protein Atlas to Establish Skeletal Muscle Specificity

The Human Protein Atlas can also be used to further screen myokine candidates for preferential skeletal muscle tissue expression, [15] which, as noted, may help to provide an extra layer of confidence in muscle as the predominant origin of production/release. The Human Protein Atlas has a defined skeletal muscle-specific proteome locatable at <https://www.proteinatlas.org/humanproteome/tissue/skeletal+muscle>, which comprises a total of 907 associated protein-encoding genes that show elevated expression in skeletal muscle compared to other tissues. The full list can be readily downloaded by first clicking on “907” in the lower right-hand corner of Table 1 from the given website link, then following the same procedure as described above for the Human Protein Atlas-defined human secretome.

5 General Myokine Experimental Considerations

5.1 *In Vivo and In Vitro Sampling*

While subject to similar experimental considerations and constraints as any life sciences experiment aimed at identifying and exploring the function of novel molecules, certain factors are noteworthy in the context of myokine-centric studies. An obvious example is whether to analyze blood versus muscle samples, with muscle being the de facto gold standard for ascertaining “true” myokines. Practical limitations clearly factor heavily into sampling choices (i.e., the technical capacity to obtain muscle biopsies) and presumably explain the literature predominance of myokine quantification in blood [22]. Systemic appearance is clearly necessary for establishing endocrine myokines. Nonetheless, given the apparent auto/paracrine predominance of myokines [5], focusing solely on blood-based myokine changes is prone to omitting a functionally important class of myokines and prevents temporal associations between muscle-blood myokine appearance, a key facet to consider when it comes to delineating molecules that reside in blood versus those secreted from cells [45]. Thus, an optimal approach to disentangling myokine function is measuring both muscle and blood, though arteriovenous (a-v) differences across muscle beds offer a strong albeit technically challenging [97] surrogate, particularly with respect to elucidating possible endocrine relevance of myokines. Indeed, a-v difference studies identified muscle as the predominant source of circulating IL-6 [16] but not BDNF [30] or FGF-21 [98], upon contraction. Measuring a-v differences in protein-containing extracellular vesicles can further inform on mechanism(s) of myokine secretion [61].

Numerous extraneous variables such as level of food intake (i.e., fasted versus fed state) and time of day (i.e., diurnal oscillations) can also affect myokine production/release [43, 99–103]. Sampling must therefore be timed accordingly and consistently to obtain reliable myokine readouts in accord with the (patho)physiological context of interest. If collecting muscle biopsies, serial samples should utilize incision sites separated at least 2.5 cm apart because the biopsy procedure may impact muscle myokine levels [104–107]. Similarly, for multiple blood withdrawals, local indwelling cannula placement is advantageous but versus venipuncture may provoke local production of certain myokines [43]. Sample storage is another consideration because several myokines (e.g., IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, and IL-15) can degrade in blood samples stored at -80°C for durations $>1\text{--}2$ years [108]. Finally, all myokine detection/quantification methods described herein can theoretically be applied to muscle tissue and blood samples. However, since blood concentrations of

secreted factors are typically very low (i.e., often pico or even femto levels) [5, 22], the greater sensitivity of ELISA/antibody arrays may be well suited for detecting/quantifying myokines in fluid samples.

Although establishing the human applicability of myokine function remains a key end goal, complementary use of a wide variety of model systems will clearly expedite myokine discoveries. Though beyond the scope here to discuss in detail the wealth of models available, transgenic animals with tissue (muscle)-specific manipulation of putative myokine levels will serve as an excellent experimental approach to establishing “true” myokines. Convincing transgenic mouse models can, however, be challenging, as with paradoxical observations of greater postexercise circulating IL-6 levels in muscle-specific IL-6 knockout mice [5, 109]. Nonetheless, if successful, such approaches will also provide a model for functional assessment of myokine gain/loss of function [45] (NB-specific analytical tools, such as MILLIPLEX® bead arrays, provide scope for species-specific myokine analyses). Moreover, because an inherent limitation of *in vivo* myokine measurement is unambiguously establishing muscle-specific myokines, *in vitro* models might be beneficial. For example, a combined examination of muscle cell cultures and associated supernatant provides a substantially simpler alternative to *in vivo* approaches for assessing myocyte-specific myokine production and secretion [23]. Frequently used muscle cell lines include the commercially available mouse C2C12 and rat L6 cell lines, as well as primary human skeletal muscle cells [24, 110]. However, key differences in transcriptomic and proteomic profiles across/between rodent/human cell lines and intact muscle [45, 111] might limit the forward translation of some newly identified myokines [23, 35].

5.2 Quantification of Myokine Gene Versus Protein Changes

A relevant factor influencing the choice of analytical approach is the relative suitability of assessing myokine protein versus mRNA levels. Owing to the presence of high-abundance proteins in blood (e.g., albumin) and muscle (e.g., contractile proteins), associated proteomes cover a vast dynamical range, in turn hindering the ability to detect low-abundance myokines using current proteomic tools [45]. Proteomic studies of skeletal muscle or plasma/serum are, therefore, generally constrained by limited proteome coverage that favors high-abundance proteins [112–114]. In contrast, established microarray and RNA-seq transcriptomic approaches offer substantially greater detection coverage, ranging from tens of thousands up to all mRNA species [115]. This, coupled with the fact that several myokines may never reach the circulation [22], perhaps implicates skeletal muscle transcriptomics-based secretome analysis as an effective initial strategy toward

novel myokine discovery [35]. Deep proteomic strategies are nevertheless emerging for greater proteome coverage of muscle/blood, though quantity of detected entities and/or capacity to detect low-abundance proteins may still be suboptimal [116–119].

Additionally, although mRNA and protein abundance often display strong agreement [114], the two can correlate poorly [45], including myokines [120]. Associations between mRNA and protein levels of secretory factors are particularly key to consider, since secretion may in some cases depend on posttranscriptional and/or posttranslational regulation (e.g., influence of specific secretory pathways, binding to neutralizing molecules, activating proteolytic cleavage, storage prior to release) [22, 33, 120]. Certainly, myokines can be regulated posttranscriptionally via translational (e.g., IL-4, IL-10, and SPARC) or posttranslational (e.g., IL-1 and IL-18) mechanisms [36, 121]. Thus, knowledge of myokine regulation within the context of the central dogma has clear implications on the relative merit of (targeted) gene- versus protein-level measurement. Toward this end, protein depletion strategies might help myokine detection [122, 123], and employing alternative in vitro models can improve proteome coverage due to attenuated dilution by high-abundance contractile proteins [45].

6 Summary: Road Map for Myokine Discovery and Analyses

In summary, there exist a number of analytical approaches that can facilitate the discovery and quantification of myokines in a variety of experimental designs. Each methodology offers distinct merits and limitations; the choice of which will be further driven by specific research goals (e.g., identifying new myokine targets versus deeper mechanistic study of a defined myokine target) and/or current state of biological knowledge (e.g., regulation of secretion pre- versus posttranscriptionally). While many defined approaches present as complementary (e.g., IHC-based localization analysis of myokines displaying altered expression via Western blot, etc.), no single method in isolation establishes myokine detection/function. Thus, resources permitting, multiple avenues should be combined [124], an analytical “road map” of which is outlined in Fig. 2. Establishing the full complexity of the muscle secretome promises exciting physiological and clinical advances, and this chapter should provide a foundation for informed experimental decisions to optimize myokine discovery and analyses.

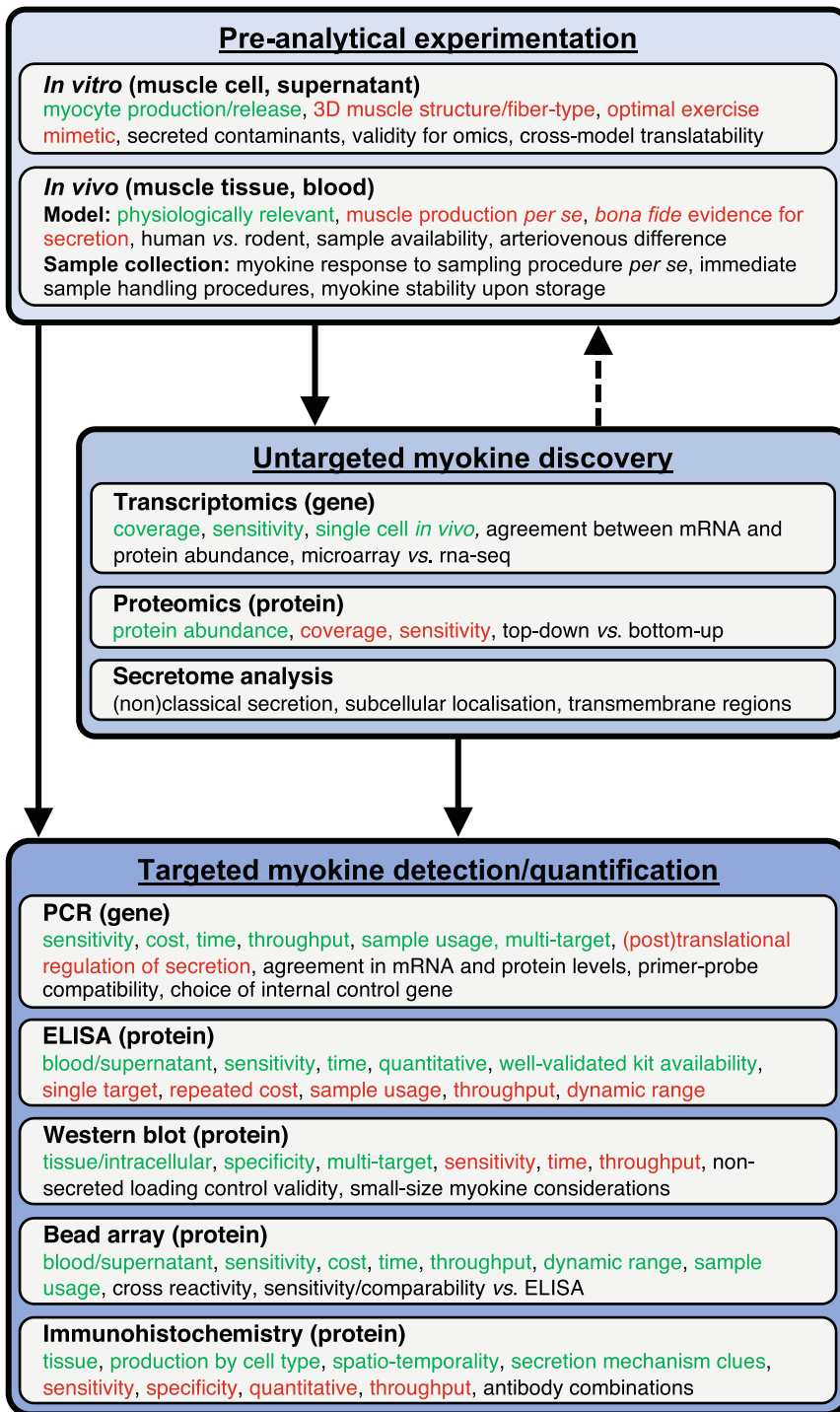


Fig. 2 Summary road map for myokine discovery/quantification. Green and red text indicate a positive/recommendable or limiting aspect, respectively (including relative to other techniques/approaches outlined). Black text indicates any other pertinent technical point worth considering in each case, as outlined at various points throughout the chapter. Filled arrows demonstrate typical analytical workflow. For example, from sample collection toward targeted detection directly or indirectly via untargeted myokine candidate identification. Dashed arrow highlights possible route for multimodel incorporation during untargeted myokine analysis phase. For example, screening for new myokine candidates *in vitro* and feeding results back toward a hypothesis-driven (targeted) experiment *in vivo* and vice versa

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