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Systems metabolomics: from metabolomic snapshots to design principles

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Metabolomics is a rapidly expanding technology that finds increasing application in a variety of fields, form metabolic disorders to cancer, from nutrition and wellness to design and optimization of cell factories. The integration of metabolic snapshots with metabolic fluxes, physiological readouts, metabolic models, and knowledge-informed Artificial Intelligence tools, is required to obtain a system-level understanding of metabolism. The emerging power of multi-omic approaches and the development of integrated experimental and computational tools, able to dissect metabolic features at cellular and subcellular resolution, provide unprecedented opportunities for understanding design principles of metabolic (dis)regulation and for the development of precision therapies in multifactorial diseases, such as cancer and neurodegenerative diseases.

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Introduction

The last decade has witnessed an increasing technical capacity to collect different omic data, including transcriptomics, proteomics, phosphoproteomics, and metabolomics. Measurement of metabolite concentrations is the more straightforward and direct approach to characterize the metabolic state of cells, tissues, and biological fluids. Recent advances in analytical techniques provide now high sensitivity and specificity. Metabolic flux analysis, in which atoms derived from stable isotope-labeled substrates are detected as they label downstream metabolic products, allows robust quantification of fluxes along biochemical pathways and can provide pathway-specific information more directly [1]. Changes in metabolic fluxes directly impact on epigenetic regulation and enzyme activities, affecting cell functions, which emerge from the network of informational and metabolite fluxes taking place in each given cell. Detailed workflows that allow quantification of metabolic rewiring in mammalian cell cultures have been recently provided ([2] and references therein).

Biological interpretation of these profiles and unambiguous identification of the affected biochemical pathways present a non-trivial task. Metabolism integrates information deriving from genetic, epigenetic, and environmental signals allowing to associate each physio-pathological condition to a specific metabolic fingerprinting [3]. We refer to the integrated use of complementary experimental and computational analyses in the study of metabolism as "systems metabolomics. This multi-layered approach allows to extract information from metabolomics and other metabolism-related data – such as transcriptomics and (phospho) proteomics – structuring it into knowledge. In the following, we review some recent advancements in systems metabolomics, outlining its use in precision medicine and wellness.

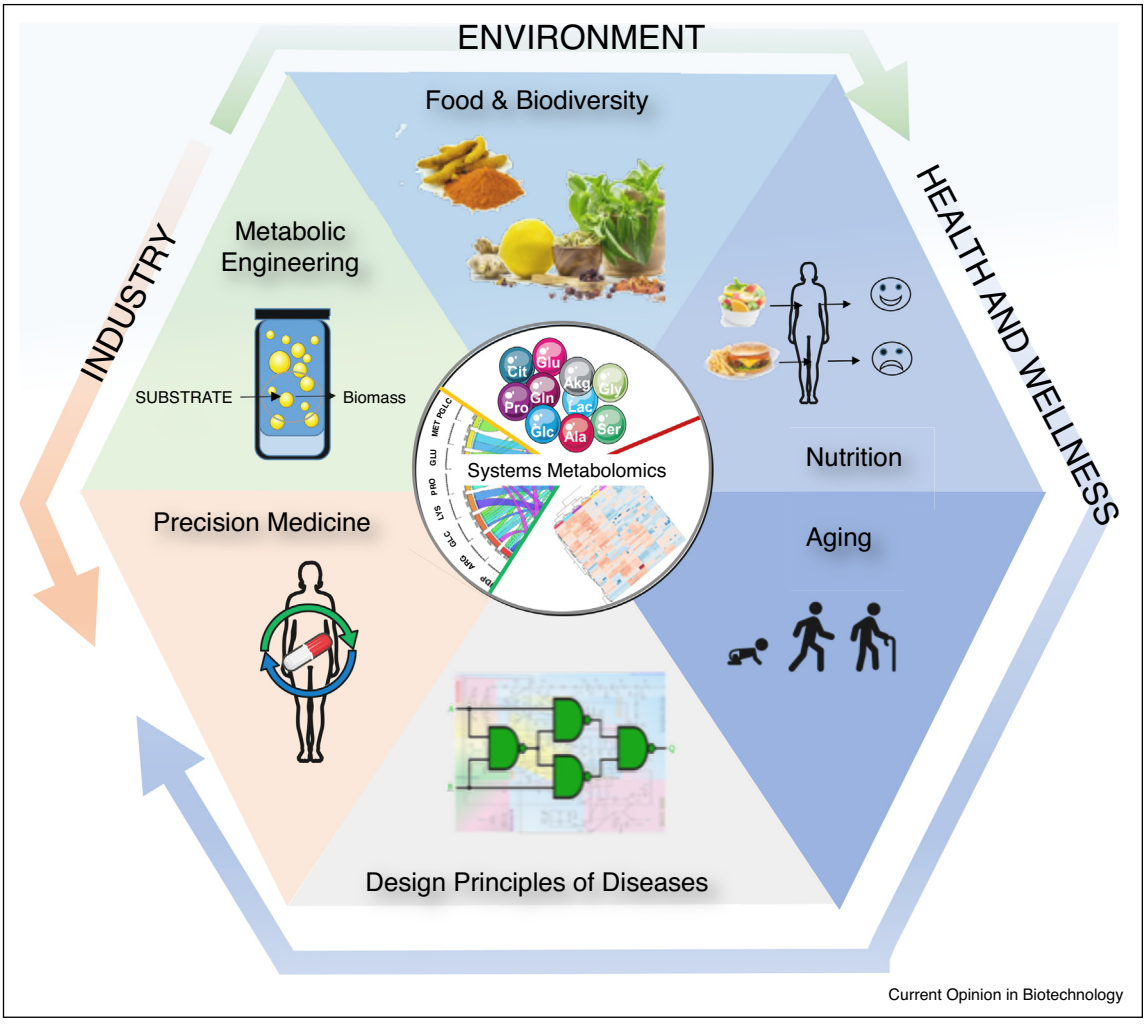
Understanding and exploiting metabolism: systems metabolomics

In recent years metabolomics has been applied successfully in multiple areas, from the discovery of novel mechanisms and new biomarkers to food and nutrition, from characterization and engineering of cell factories to the health and clinical field (Figure 1) [4–7,8••,9].

Metabolome and fluxome data are essential to understand the metabolic status of the producer strain during fermentation and identify engineering targets to enhance its performance further. Accordingly, emerging and successful strategies for developing industrial microbial strains, that is, cell factories capable of overproducing bioproducts, are based on system-wide metabolic engineering and optimization and require metabolomics analysis and genome-scale computational simulation [10,11].

In nutrition and wellness, complex metabolomics data from biological samples, such as saliva, blood, urine, and cerebrospinal fluid, are being used to discover novel biomarkers of dietary intake, host-environment interaction,

Figure 1



The central role of systems metabolomics in different fields.

acute and chronic physical activity, stress, and other extrinsic factors affecting human health and disease [12–14]. Metabo-typing of individuals is becoming an important assessment tool that allows customization of nutritional requirements to obtain the best possible outcomes from precision nutrition [15], as a function of different lifestyles and critical illness (such as myopathy, cardiomyopathy, and neuropathy) [16]. Metabolomics may directly assess the nutritional composition and authenticity of food, plant varieties, and crop cultivars, information which are indispensable to both consumers and food producers who take an interest in potential health benefits and nutritional value of foods seen as 'nutraceuticals' [17].

In the health field, many pathophysiological states include a metabolic component. A general rewiring of energy metabolism is a hallmark of aging [18,19], neurodegenerative diseases [19–21], and cancer [22,23]. Otto Warburg reported the first evidence of an altered metabolic phenotype in cancer a century ago [24]. Recent evidence shows that metabolic rewiring occurs both as an indirect consequence of oncogenic mutations, as well as by direct mutation of genes encoding metabolic enzymes, such as the genes encoding the tricarboxylic acid cycle enzymes Fumarate Hydratase, Succinate Dehydrogenase, and Isocitrate Dehydrogenase. These events may lead to the accumulation of metabolites – referred to as *oncometabolites* – that can act as oncogenic signaling molecules [25]. Interestingly, opposite transcriptional modulation of the genes encoding Isocitrate Dehydrogenase contributes to cancer and neurodegeneration [26].

A physiological, system-level analysis of metabolism – obtained through high-resolution respirometry (Oxygraph 2k, Oroboros Instruments) [27] and extracellular flux analysis (Seahorse eXtracellular Flux (XF) analyzer,

Agilent) – allows to characterize the overall metabolic phenotype of cells (cultured as adherent monolayers (2D) or spheroids (3D) in a multi-well plate format) and tissues. Seahorse technology measures simultaneously mitochondrial respiration and glycolysis and their dependence on drug and nutritional stress, putting in context data obtained by metabolomics, drug sensitivity, or labeling experiments [28–31].

As witnessed by the ever-increasing application in clinical cancer research [22,32,33], the use of metabolomics in personalized medicine aims to identify the most effective treatment in patients based on their response towards particular drugs [34] and guide the development of more effective drugs by providing a better understanding of the system-wide effectiveness they have *in vivo*.

Metabolic modeling meets artificial intelligence

Computational tools are needed to contextualize experimental observations into a systems-level understanding of metabolic alterations. By way of example, information on enzyme concentrations and metabolomics must be translated into information on metabolic flux distributions, whose experimental determination may not always be feasible, especially in a clinical setting.

The application of Artificial Intelligence to this problem has so far remained sporadic, mainly due to the large number of data required for efficiently training machine learning algorithms, on the one side, and to the limited access to public metabolic data on the other side [35•]. Existing studies mainly attempt to predict metabolomes from proteomic [36] or multi-series omic data [37] or the metabolic flux of a small portion of a network, such as the upper part of glycolysis [38]. Moreover, machine learning algorithms are characterized by the well-known black box problem, which makes it difficult to know how and why they work [39].

Constraint-based metabolic network modeling has been more successful in providing a mechanistic understanding of metabolism, providing predictions of changes of fluxes as a function of nutrient supply. Genome-Wide Metabolic Models (GEMs, [40]) include thousands of reactions and describe genotype-to-phenotype association mechanistically, through Gene-Protein-Reaction (GPR) associations. Core models include a reduced number of reactions and may help in the identification of the design principles governing each metabolic pattern rewiring. Constraint-based simulation of a core model describing glucose and glutamine conversion into biomass [41] showed that when available oxygen is not sufficient to fully oxidize available glucose and glutamine carbons, a situation often observed even in normoxia, reductive carboxylation of glutamine, and conversion of glucose and glutamine to lactate, confer an advantage for biomass production. GEMs (and core models) provide cellular context for omic data analysis, enabling to go beyond simple statistical correlations [42,43], or standard machine learning methods [44,45]. In the more typical and straightforward case, metabolomic data of spent culture media are used to derive constraints on extracellular fluxes [46].

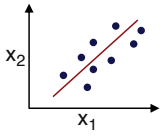
The steady-state assumption and constraints on extracellular fluxes do not result in a unique steady-state flux distribution. For this reason, many algorithms have been proposed for the integration of other high-throughput data to further reduce the solution space of feasible flux phenotypes, with particular regard to transcriptomic data. These algorithms may map transcriptomic data to topological information in GEMs to enrich for metabolites [47] or metabolic reactions [48]. Other algorithms extract a subnetwork of the GEM that corresponds uniquely to active reactions in the context of interest or predict metabolic flux distributions. This objective is achieved by incorporating data into constraint-based simulations, either providing constraints on the values of admitted fluxes (e.g. scFBA [49•], eFlux [50]) or leading to the formulation of an objective function that seeks to minimize the distance between predicted flux distribution and data (e.g. *GIMME* [51], *tINIT* [52], *iMAT* [53]). Most of the latter algorithms have been reviewed and benchmarked in Refs. [54•,55•,56].

Metabolomic data are more tightly related to metabolism than transcriptomic data, but their integration is more challenging (Figure 2). Some methods ask the network to produce specific metabolites at the stage of context-specific network reconstruction or require demanding time-series data [57,58]. iReMet-Flux integrates metabolomic data in a metabolic model predicting fluxes in a way that complements methods based on radioactive tracer labeling [59]. More recent studies – reviewed in Ref. [58] – attempt to directly incorporate knowledge on relative changes (after a perturbation) in metabolite abundances as model constraints [57,58,60•].

Approaches coupling metabolic modeling with artificial intelligence use the mechanistically linked information provided by context-specific models as the input of either supervised or unsupervised machine learning approaches, as reviewed in Refs. [61–63]. Unsupervised approaches typically aim at partitioning condition-specific metabolic networks or flux distributions, based on intra-cluster similarity and inter-cluster dissimilarity, and/or at reducing their dimensionality to essential components, retaining only features that are responsible for the variability in the data. A recent example uses principal component analysis on a thousand cancer genome-scale metabolic models [64]. The work shows that tumor metabolic networks differ more in the expression of specific enzyme-encoding genes than in the structure of the network itself. After training an algorithm with many examples of model-

Figure 2

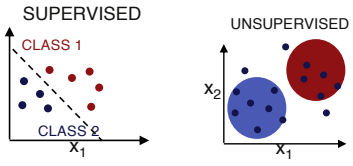
STATISTICAL MODELS



[42]
Correlation between consumption of metabolites or expression of enzymes and proliferation rate.

[43]
Identification of transcript-metabolite abundance pairs whose correlation significantly differ between phenotypes (e.g., blood cancer and solid tumor cell lines).

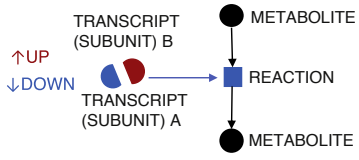
MACHINE LEARNING



[44]
A machine learning classifier (supervised learning) was trained with NCI60 data (including growth rates) to predict the cell growth of tumor samples from gene expression data.

[45]
Unsupervised clustering of 3D Mass Spectrometry Imaging data to identify the presence of tumor subgroups of cells characterized by similar mass spectrometry profiles.

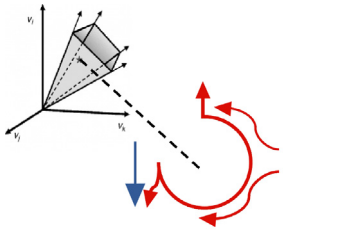
KNOWLEDGE-BASED STATISTICS



[47]
Metabolites are enriched between two conditions according to expression of neighbouring enzymes.

[48]
Metabolic reactions are assigned scores according to the expression of enzyme isoforms and subunits. Significantly different scores between phenotypes are mapped onto metabolic networks.

CONSTRAINT-BASED MODELING



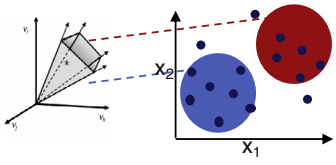
[46]
Extracellular fluxes as constraints to predict flux distribution of NCI-60 cancer cell lines.

[54]
Extracellular fluxes and transcriptomics data as constraints to improve predictions on NCI-60 cancer cell lines, such as proliferation rates, gene essentiality, drug response.

[59]
Relative changes in metabolite abundances as constraints in a quadratic programming problem.

[57]
Integration of time-resolved metabolomics and transcriptomics data.

MACHINE LEARNING + CONSTRAINT BASED MODELING



[62]
Context-specific metabolic models are reconstructed with FASTCORE. Supervised machine learning (feature selection) is then used to identify the set of genes that more discriminate healthy from cancer models.

[65]
A classifier based on support vector machines is trained to segregate between drugs that are likely to be associated with side effects based on their effect on predicted metabolic flux distributions.

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Families of modeling and artificial intelligence approaches to systems metabolomics and explicative examples.

property pairs, supervised approaches more often aim to identify a function that correctly predicts a phenotypic property associated with any metabolic network or flux distribution. For instance, Shaked *et al.* [65] built a classifier that determines whether or not a drug causes side effects based on constraint-based simulations of the inactivation of the drug's targets. These studies typically also exploit feature selection algorithms to identify the set of features (genes, reactions, or pathways) that improve the accuracy of the predictor. In Ref. [62], the identification of genes more relevant for segregating between cancer and healthy models leads to the identification of new drug targets. In Ref. [66••], the simulated metabolic fluxes that mostly determine the level of the concentration of antibiotic needed to inhibit bacterial growth provided novel indication on pathways involved in antibiotic resistance.

Multi-omic and single-cell analysis of metabolism

New computational frameworks are being developed to exploit the increasing availability of multi-omic data. They can be used, for instance, to better identify a metabolic signature capable of predicting patient survival integrating metabolomics, lipidomics, and transcriptomics [67], to improve patient stratification via multi-view clustering algorithms (such as Ref. [68], as reviewed in Ref. [69]), to discover biological pathways with correlated profiles across multiple complex data sets [70].

GEMs offer a knowledge-based opportunity to integrate the different sources to provide a unified vision of the systems under study. For example, [60•] incorporates the determination of the relative levels of both metabolites and transcripts. A recent study [71••] combines multi-omic data integration with metabolic network and machine learning to recapitulate the regulation of breast cancer cell metabolism.

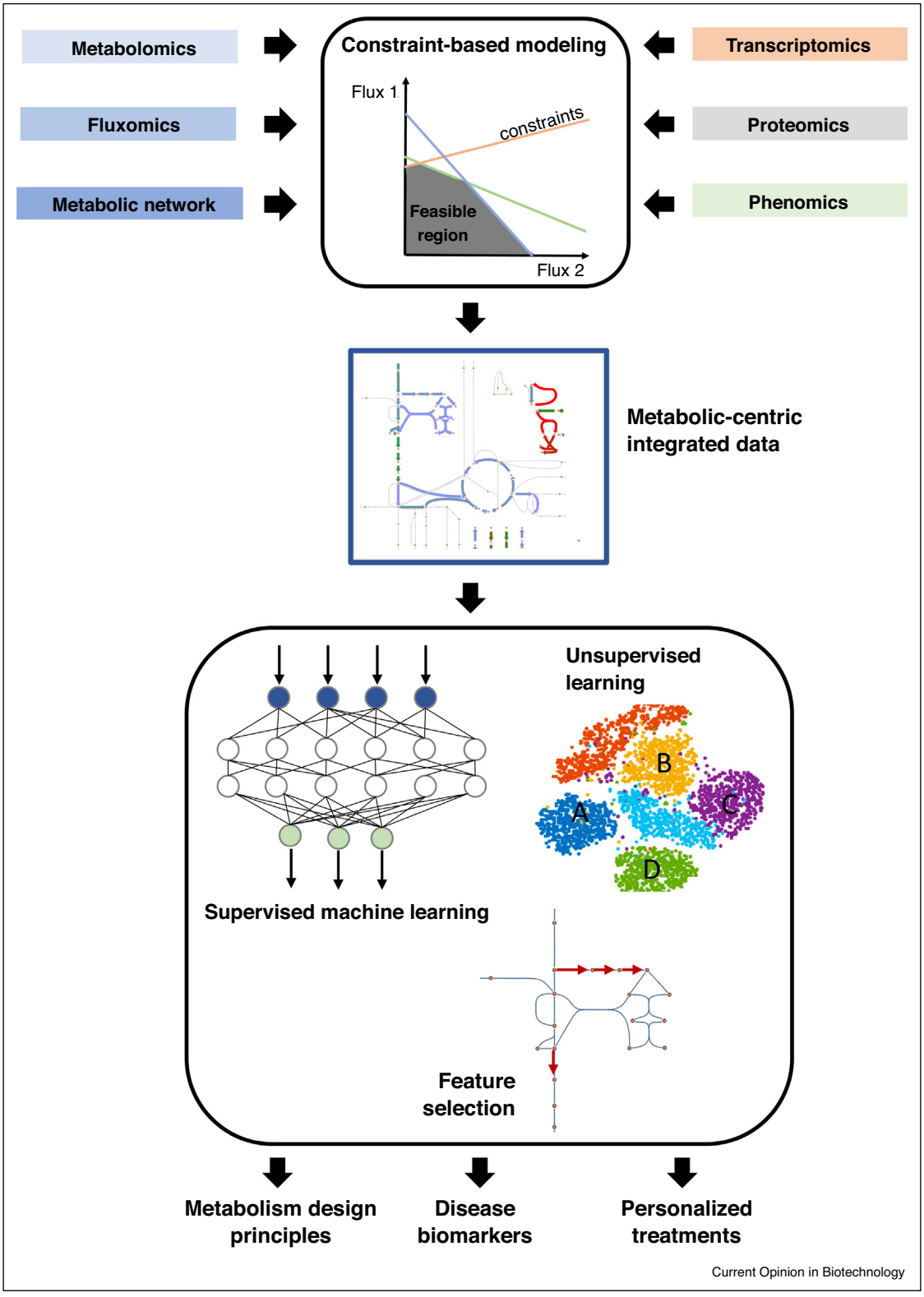
A major challenge is now posed by metabolic heterogeneity at the intra-tissue level. Many tissue metabolic functions – and dysfunctions – rely on interactions between cell types. For example, the metabolic interplay that occurs among cancer and stromal cells contributes to cancer development and resistance to treatment. Thus, multicellular, constraint-based models are being proposed that integrate the metabolic activities of multiple cell types, such as Refs. [72,73] and other papers reviewed in Ref. [74].

Single-cell omics allow phenotypic characterization of cells at high resolution. Single-cell metabolomics poses significant challenges because of the low analyte abundances and limited sample volumes [75]. With its high spatial resolution, imaging mass spectrometry, can map and quantify a wide range of unlabeled small molecules [76••,77,78] review some applications in the field of cancer and neurodegeneration, respectively.

High Content Analysis (HCA, or cell-omics) correlates high-resolution spatial information with quantitative parameters, including the level of specific metabolites, or the expression and activity of specific metabolic enzymes or their regulators, using in vitro biochemical assays [79]. Many HCA studies of metabolism use specific fluorescent probes on live cellular models (cell lines or primary cells, cultured in 2D or 3D, tissue samples, and organoids). Potentiometric and non-potentiometric mitochondrion-selective probes allow studying parameters strictly related to the mitochondria function (fission, fusion, fragmentation, membrane potential) [80]. Probes measuring mitochondrial or cytoplasmic reactive oxygen species (ROS) [81] and genetically encoded ratiometric fluorescent sensors specific for pyridine nucleotides, such as NADH, NADPH or thiols [82,83] allow measuring redox homeostasis. Of particular interest is the possibility to study the redox state of live cells by taking advantage of the autofluorescence of specific metabolites, such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), flavin adenine dinucleotide (FAD) that allow for label-free quantification of metabolic activity of individual cells over time and in response to various stimuli [84,85]. The multiparametric nature of HCA allows correlating the cellular metabolic status or the response to specific metabolic perturbation in real-time and at the single-cell level to many important phenotypic traits, including cell proliferation and apoptosis, autophagy, cell migration or invasion.

The collection and analysis of single-cell RNA sequencing (scRNA-seq) data have significantly advanced and increased our knowledge of gene expression heterogeneity [86], but little application to metabolism have so far been reported. Single-cell Flux Balance Analysis [49•] allows translating single-cell RNA profiles into single-cell fluxes. The exchange of metabolites among cells allows the identification of possible inter-cellular metabolic interactions and to cluster cells according to the growth rate, a parameter strongly related to the tumor aggressiveness. This kind of analysis does not directly provide information on the spatial distribution of cells. FBCA (Flux Balance Analysis with Cellular Automata), couples biomass accumulation (simulated via Flux Balance Analysis of metabolic networks), with the simulation of spatial dynamics (via Cellular Potts Models) of cell populations that communicate through metabolite secretion [87]. Integration of FBCA (extended in 3D, compared to actual 2D analysis) and scFBA may provide the foundation for determining spatially resolved single-cell fluxes of heterogeneous cell populations in clinical and pre-clinical models. Using a similar approach that combines metabolic modeling with cellular automata, Shan *et al.*, provided a multi-scale model that stresses the importance of taking into account spatial and temporal evolution of the tumor microenvironment for a complete understanding of different metabolic scenarios such as the Warburg and reverse Warburg effects or glutamine addiction [88].

Figure 3



A flow chart for a systems metabolomics approach applied to personalized therapy of multifactorial diseases.

Conclusions and perspectives

Changes in the levels of metabolites and their cognate fluxes are sensitive readouts of the response of biological systems to genetic and/or environmental perturbations. These changes result from the complex, non-linear interaction of metabolic, signaling, and regulatory pathways. A broad spectrum of techniques allow accurate and sensitive measurement of metabolites and metabolic fluxes. Cytological, high content, and physiological techniques allow the overall characterization of the physiological state of cells and tissues as well as their dependence on specific nutrients. Constraint-based metabolic models provide a framework for the integration of high-throughput data, such as population and single-cell transcriptomic data, as well as multi-omic data [89].

Systems metabolomics combines these different experimental and computational approaches aiming to unravel the design principles of metabolic regulation. In molecular oncology, systems metabolomics studies the interconnection of metabolism with physio-pathologically relevant properties, such as proliferation [3,41,52] or metastatic dissemination [90,91••], with the final aim to provide the rational basis for the development of individually tailored precision oncology therapeutic strategies. It can contribute to the development of pre-clinical pipelines that incorporate information derived by systematic perturbation analysis of experimental models of cancer patients — including patient-derived cell lines, spheroids, organoids, and organs-on-chip, and xenotransplants (PDXs). Since integrated analyses are costly, time-consuming, and often unfeasible in a clinical setting, a primary goal of systems metabolomics is to derive appropriate proxies to be used in the design of combinatorial drug treatments as well as in the therapeutic follow-up (Figure 3).

By similar reasoning, in nutrition and wellness, a personalized systems metabolomics approach would provide a complete picture of the functioning of the person's metabolism, allowing a prediction of the effects that the administration of particular diets would have on the organism. The identification of the design principles connecting nutrient utilization to metabolic functioning and the organism's ability to cope with environmental insults would lead to the rational formulation of appropriate alimentary regimens tailored for the wellness of each individual according to the various phases of a person's life and her/his 'lifestyle'. Such a rational approach can actively contribute to preventing disease development, better support people under pharmacological treatments, entailing an enormous advance in terms of both the prevention of diseases and an improvement in the quality of life.

Author contributions

Lilia Alberghina discussed the review idea and revised the paper.

Chiara Damiani, Daniela Gaglio and Elena Sacco wrote the paper, conceived, and prepared the figures.

Marco Vanoni conceived the review idea, discussed the images, wrote and revised the paper.

Conflict of interest statement

Nothing declared.

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