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Metabolic cell communication within tumour microenvironment: models, methods and perspectives

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Environmental cues are essential in defining tumour malignancy, by promoting tumour initiation, progression and metastatic spreading. Stromal cells may metabolically cooperate or compete with cancer cells, playing a mandatory role in defining cancer metabolic plasticity, potentially dictating the final tumour outcome. Assessing shared nutrients between different tumoural or stromal compartments is essential to understand the impact of environmental nutrients on the metabolic plasticity of tumours. Here, we review analytical and computational approaches for studying the tumour metabolic microenvironment, the destiny of nutrients shared among tumour and stromal populations, as well as the molecular modules of these metabolic relationships.

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Introduction

Beside tumour cells, tumour microenvironment (TME) is composed by several stromal cells, including cancer associated fibroblasts, endothelial, adipose and immune cells, that assist cancer cells during their progression towards malignancy. Among these populations a biunivocal relationship is rapidly established, where cancer cells secrete several factors in the so called 'afferent way', and stromal cells are therefore 'educated' to answer with secretion of other soluble molecules ('efferent way'), which in turn assist cancer cells to enhance aggressiveness. These soluble molecules, either hormones, cytokines, small RNAs or nutrients can be exchanged directly or encapsulated into microvesicles. Table 1 summarizes the current available data concerning exchanges within TME of nutrients and macrovesicles (Figure 1). An accurate assessment of metabolic cross-talk between tumoural and stromal compartments is challenging, as the nutrient net transfer versus the exchange flux are poorly distinguishable with existing biochemical methodologies. This review is focused on the assessment of how microenvironment, particularly the soluble part (which is the most shared between tumour-associated cell populations) of its metabolic enrichment, affects tumour metabolic phenotypes through the development of new emerging technologies, able to metabolically characterize not only *in vitro*, but particularly *in vivo* and *ex vivo* models of cancer likely representing good models of metabolic tumour-stroma interplay.

New molecular approaches in the analysis of metabolic exchanges

Significant advances in analytical methods that support the investigation of cellular metabolic nutrients exchanges have furthered the discovery and characterization of metabolic programs critical for tumorigenesis.

NMR and Chromatography coupled to mass spectrometry

Metabolomics of cell-cell communications reflects the study of metabolism in a comprehensive way. Multiple analytical platforms that facilitate the detection of metabolites have been utilized to study cancer-associated intrinsic and microenvironmental metabolites [49,50]. Since many of the metabolites associated with nutritional exchanges within TME, have peculiar chemical properties, appropriate analytical platforms that can measure these metabolites are essential to understand cancer promoting metabolic pathways [51]. In particular, chromatography (gas chromatography (GC) and liquid chromatography (LC)) are used for metabolite separation. When coupled to mass spectrometry (MS) detectors, these techniques represent the most powerful methods used to determine the metabolite profiles of such biological samples [52] (Table 2). Another quantitative analytical technique used for metabolite analysis is nuclear magnetic resonance (NMR). NMR detects molecular features by measuring the spin, an intrinsic magnetic property of atomic nuclei, giving data about the quantity, structure, reaction state, and chemical environment of molecules. Attempts have been made to integrate these different analytical platforms (LC–NMR–MS) [53]. MS is more commonly used for untargeted metabolite analysis since it is more sensitive, and can distinguish and quantify more molecules in complex biological samples, in which soluble nutrients exchanged between cells are found. Two frequently used methods for MS metabolites

Table 1

Nutrient-based exchanges in the tumour microenvironment. Soluble and vesicular mediators of functional and metabolic interplay between various kind of cancers and their stromal environment

Nutrient/Cargo	Donor cells	Recipient cells	Effect
Lactate	CAFs	Cancer cells	1 activation of epithelial-to-mesenchymal transition, achievement of stem cells traits and enhanced metastatic spread [1,2] 2 metabolic reprogramming towards OXPHOS, increase of mitochondrial function [3••,4,5] and TCA fueling [3••,4,5] 3 immunomodulatory activity towards Th1 and Treg cells [6]
	Cancer cells	CAFs	Causes strong adaptive resistance to MET and EGFR targeted therapies [7]
	Cancer cells	Immune cells	Activation of lactate-sensible GPR81 GPCR coupled receptor and immune response through upregulation of PD-L1 [8]
	-	Neuronal cells	Activation of lactate-sensible GPR81 GPCR coupled receptor and tuning down neuronal network activity [9]
	-	Adipocytes	Reduction of lipolysis via GPR81 lactate receptor [10]
Succinate	Cancer cells	Endothelial cells	Cell proliferation and migration; VEGF secretion; capillary formation and development of new vessels formation; achievement of stem cell traits [11]
	-	Cancer cells	Uploaded via SLC13A3 plasma membrane carrier leads to enhanced prostate cancer malignancy [12]
	Macrophages	Neural stem cells	Secretion of prostaglandin E2 with anti-inflammatory effects [13]
	Macrophages	Immune cells	Uploaded via SLC13A3 plasma membrane carrier fuels inflammation in an autocrine manner, sustaining and amplifying the inflammatory response [14]
	Cancer cells	Macrophages	Shifts towards M2 macrophage polarization and enhances cancer malignancy via interaction with is membrane GPCR SUCNR1 [15]
Citrate	TME	Cancer cells	Uploaded via its plasma membrane variant of the SLC25A1, it redirects tumour metabolism towards lipogenesis and affect cancer malignancy [16]
Pyruvate	CAFs	Cancer cells	Fuels TCA cycle of primary lymphoma cells and supports their survival [17]
Glutamine	TME	CAFs	Allows metabolic flexibility and adaptive mechanisms in stromal cells, supporting cancer growth and metastasis [18]
	Adipocytes	Pancreatic cancer cells	Cancer cells proliferation [19]
	Astrocytes	Glioblastoma cells	Allows fueling *de novo* purine biosynthesis and supports glioblastoma progression [20]
	TME	Macrophages	Supports M2-macrophages polarization associated with a decreased ability to recruit immune and endothelial cells [21]
Alanine	Pancreatic stellate cells	Cancer cells	Outcompetes glucose/glutamine-derived carbons to fuel the TCA cycle, supports lipid and non-essential amino acid synthesis [22]
Aspartate	CAFs	Cancer cells	Feeds TCA cycle by sustaining the pyrimidine biosynthesis in cancer cells exposed to enhanced ECM stiffness [23•]
Glutamate	Cancer cells	CAFs	Allows to maintain redox homeostasis in CAFs through glutathione biosynthesis [23•]
Kynurenine	CAFs	Cancer cells	Engages EMT and malignancy, leads to immune suppression through regulation of Th1 and Th2 cells [24]
Free Fatty Acids (FFAs)	Adipocytes	Melanoma cells	Uploaded through CD36, they enhance invasion and melanoma cell growth [25]
	Adipocytes	Ovarian/ melanoma Cancer cells	Uploaded through CD36, they promote tumour chemoresistance and progression [26,27]
	Adipocytes	Breast cancer cells	Activation of a reciprocal interplay in which Cancer cells activate lipolysis in adipocytes and FFA causes enhanced proliferation and invasion of cancer cells [28]
Mitochondria	Stromal cells	Mitochondria-defective cancer cells	Rescue of a respiratory behavior [29,30]
	Bone marrow-derived (MSCs)	Leukemic blasts	Increase in mitochondrial mass, OXPHOS and ATP production as well as the drug resistance of cancer cells [31–33]
	CAFs	Prostate cancer cells	Enhance OXPHOS metabolism, ROS production and invasiveness in cancer cells [5]
miRNA (exosomes)	Pancreatic tumour cells	Pancreatic tumour cells	Chemotherapeutic drug treatment induces miR-155 expression in cancer cells. miR-155, which possess anti-apoptotic function, is then delivered, via exosomes, to other cells of the tumour contributing to chemoresistence [34].
	CAFs	Head and neck cancer cells	miR-196a, delivered from CAFs to tumour cells via exosomes, confers cisplatin resistance to head and neck cancer cells [35]
	CAAs or CAFs	Ovarian cancer cells	miR21, transferred from CAAs or CAFs to the cancer cells, suppresses ovarian cancer apoptosis and confers paclitaxel chemoresistance [36]

Table 1 (*Continued*)

Nutrient/Cargo	Donor cells	Recipient cells	Effect
	Bone marrow-derived mesenchymal stem cells (BMSCs)	Lung cancer cells	Exosomes, produced by hypoxic BMSCs, promote cancer cell invasion and EMT by transferring miR-193a-3p, miR-210-3p and miR-5100 to cancer cells [37]
	Pancreatic tumour cells	TAMs	Exosomes containing miR-301a, produced by hypoxic pancreatic cancer cells, activate macrophages to the M2 phenotype, which, in turn, facilitates the EMT of cancer cells [38]
	Gastric cancer cells	Gastric cancer cells	miR-501, overexpressed in doxorubicin-resistant gastric cancer, is delivered via exosomes conferring doxorubicin resistance to the other cancer cells of the tissue [39]
	Glioma cells	Glioma and vascular endothelial cells	miR-9, overexpressed in glioma cells, is secreted via exosomes and then absorbed by vascular endothelial cells or other cancer cells of the tissue, promoting angiogenesis and metastatic invasion [40]
	Glioma cells	Myeloid-derived suppressor cells (MDSCs)	Hypoxic glioma cells stimulate the differentiation of MDSCs by transferring exosomal miR-29a and miR-92a, leading to an immunosuppressive response within TME [41]
Proteins (exosomes)	Melanoma	Bone marrow cells	Exosomes from metastatic melanoma cells 'educate' bone marrow progenitors through the receptor tyrosine kinase MET, increasing the metastatic behavior of primary cells [42]
	Gastric cancer	Liver cells	Epidermal Growth Factor Receptor in exosomes secreted from gastric cancer is delivered into the liver inducing a favorable environment for cancer cells metastatization [43]
	Prostate cancer	Mesenchymal stem cell (MSC)	Exosomes, secreted by prostate cancer cells, induce a TGF-β mediated differentiation in bone-marrow MSC favoring cancer proliferation and invasiveness [44]
	Adenocarcinoma cells	Endothelial cells	Exosomes containing tetraspanin produced by adenocarcinoma cells promote neo-angiogenesis [45]
Metabolites (exosomes)	CAFs	Cancer cells	CAF-derived exosomes contains amino acids, lipids, and TCA-cycle intermediates that are utilized by cancer cells for their metabolism promoting tumour growth [46]
Bioactive lipids (exosomes)	TAMs	Cancer cells	TAM exosomes contain bioactive lipids and biosynthetic enzymes, which promote pro-inflammatory signaling in cancer cells [47]
Proteins (Microvesicles)	CAFs	Prostate cancer cells	Microvesicles, produced and released by CAFs, transfer many proteins to cancer cells, including Galectin-1, Superoxide dismutase, Malate dehydrogenase, Vimentin, Pyruvate kinase M2, that lead to an increased cancer cells proliferation rate [48]

analysis, coupled to the LC/GC chromatography, are multiple reaction monitoring (MRM) and high-resolution MS (HRMS). MRM experiments are usually conducted on a triple-quadrupole mass spectrometer [54]. HRMS rely on the high mass resolution of the mass analyzer. One frequently used mass analyzer is the Orbitrap, which records the time oscillation of ions, the frequency of which provides information on the molecular mass [55]. Another is a time-of-flight (TOF) instrument, which records the time needed by an ion to cross an electric field [56]. These mass analyzers greatly simplify soluble metabolites identification compared with lower-resolution methods. In addition, the amount of material required for metabolomics analysis with most recent MS/NMR technologies is becoming smaller, which allows single-cell metabolite profiling within cells, conditioned media, tissues and extracellular vesicles (EVs) [57].

GC-MS is ideally suited for the analyses of compounds that are non-polar, volatile and nonvolatile, and have a small molecular weight. The compounds are generally separated by different boiling points. The high resolution and reproducible chromatographic separations offered by modern GC-MS make it an excellent tool for complex metabolic mixture analyses. LC-MS is applied mainly for the analysis of thermally unstable molecules in complex samples. LC-MS is a separation performed in the liquid phase and which are incompatible with GC. The sample type and the metabolites of interest determine the appropriate sample preparation procedures [58–60].

Attention in EVs has grown greatly in recent years due to the remark that these vesicles deliver a novel means of communication among distant cells and this happens through the exchange of miRNA, proteins, metabolites and lipids [61]. The first prerequisite is the obtainability of standardized and contamination-free vesicle productions. The current conventional proteomics workflow is founded on nano-HPLC joint on-line with tandem mass spectrometry (nano-HPLC-MS/MS) [62]. This analytical tool can find hundreds or even thousands of metabolites, lipids and proteins in a EVs. Compared to the central role of MS in EVs proteomics, at present MS-based workflows are less commonly used for lipidomic researches of EVs. Most publications discovery diverse lipid classes with thin layer chromatography [63]. Some investigators have

Table 2

Analytical platforms for metabolic assessment of cell-cell communication in the tumour environment

	Advantages	Disadvantages	Applications	Ref
LC–MS	• Wide metabolite analysis • High sensitivity • Many open-source software assisting data analysis	• Not quantitative • Destructive	• Metabolomics analysis • Metabolic Flux Analysis (MFA)	[36,49,51,52,53]
GC–MS	• Wide metabolite analysis • High sensitivity • Many open-source software assisting data analysis	• Not quantitative • Not appropriate for nonvolatile or thermally fragile molecules • Difficulties from multiple derivatization products from a single metabolite	• Metabolomics analysis • Metabolic Flux Analysis (MFA)	[36,61]
NMR	• Wide metabolite analysis • Many open-source software assisting data analysis • Quantitative	• Low sensitivity • Fewer metabolite coverage per run • High cost of equipment and maintenance	• De novo compound structure analysis	[38]
High-Resolution Respirometry (HRR)	• Small amounts of cells or tissue • Stability and quality control • Low cost	• Extremely delicate instrument	• Mitochondrial studies in cell physiology and pathology	[69]
Seahorse XF Analyzers	• Measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of live cells	• High Cost of disposable material	• Simultaneous assessment of glycolytic and oxidative metabolism	[70,71]
Genetically encoded FRET fluorescent biosensors	• Real-time detection of metabolites	• Needs an accurate analysis of the fluorescence signal	• Laconic, Pyronic and FLIPE can detect lactate, pyruvate and glutamate respectively	[65,66,67]
Induced metabolic bioluminescence imaging (imBI)	• Measure key metabolites in tumour sections	• The samples are extremely delicate	• Spatial resolution of metabolites directly in the tissue	[68]

utilized methods such as HPLC [64]. LC-MS platforms for EVs lipid characterization have been applied only in two articles [65,66].

Metabolite tracing

The overall metabolite profile is informative in many cases, but for metabolites involved in multiple catabolic and anabolic pathways, metabolite steady state concentration is the results of many "afferent" or "efferent" pathways. An appropriately designed isotopic tracing study is then used to identify the contribution of each pathway to the steady state level of a given metabolite. Carbon ^{14}C labeled nutrients can be used for *in vitro* metabolic analysis in order to detect the ability of cells to exploit such nutrient in terms of uptake and respiration, as well its incorporation in different macromolecular pools (lipids, DNA, proteins). These assays are useful to resemble the *in vivo* cell-to-cell nutrients' feeding/shuttling and how the environmental molecules can impact on the metabolism of recipient cell [5]. Other type of experiments that use ^{13}C heavy stable isotopes are utilized to represent the metabolic flux from different sources [67]. Protocols include incubating a stable isotope-labeled ^{13}C nutrient (such as glucose, an amino acid, a lipid, or other molecules) at the equivalent concentration as the original experiment and waiting for the metabolic flux to reach steady state [68,69]. These procedures are together called 'metabolic flux analysis' (MFA) and include taking a series of high resolution MS isotope (isotopologues) measurements of different metabolites, overlaying them on a metabolic network, and fitting a mathematical model of the fluxes on the network that best fits the data. Recently,

with the support of stable-isotope tracers and network analysis, other metabolic pathways activated in cancer cells have been identified. These include changes in metabolite abundances induced by CAF exosomes in cancer cells [46], reductive metabolism of glutamine [70], altered glycolysis [71], serine and glycine metabolism [72], acidic amino acid metabolism [73], one-carbon metabolism [74] and acetate metabolism [75].

Genetically-encoded fluorescent biosensors

LC–MS, GC–MS, NMR techniques need long run-up times prior to the detection of metabolite levels which limits the practical number of samples suitable for analysis in each experimental run, thus the number of observable time points for an experiment is limited when multiple time points are mandatory to assess metabolic network. To overcome these limitations, genetically encoded fluorescent FRET biosensors have been developed to facilitate the real-time detection of metabolites. In a FRET biosensor, when the metabolite binds the sensor, a conformational change that permits FRET between fluorophores occurs. For example, Laconic, Pyronic and FLIPE can detect lactate, pyruvate and glutamate respectively [76–78]. Biosensors have improved the evaluation of dynamic flux and metabolite exchange between cells and their environment.

ImBI

Induced metabolic bioluminescence imaging (imBI) is a useful imaging technique that enables detection of key metabolites, including lactate, glucose, pyruvate, and ATP in tumour sections. ImBI provides an accurate representation of the levels of the metabolites, distinguishing glucose, lactate or pyruvate cellular content. The sample preparation needs self-made kits containing definite exogenous enzymes and cofactors, specific for glucose, lactate or pyruvate, finally detected by bioluminescence. The following step is the matching to the histology of the tumour by a sandwich technique and the combination of the signals of bioluminescence and hematoxylin/eosin in serial tissues cryosections [79]. This emerging technique allows the spatial resolution of metabolites directly in the tissue.

High-resolution oxygraph

Measurements of cellular respiration provide important insights into mitochondrial respiratory capacity, mitochondrial integrity and energy metabolism. One of the devices which enable measurements of mitochondrial oxygen consumption with high accuracy, resolution and sensitivity is the high-resolution oxygraph Oroboros O2K (Oroboros instruments) [80]. With the Oroboros O2k, small amounts of biological samples can be used for bioenergetic analysis, ranging from isolated mitochondria to intact cells that can be treated with specific drugs and nutrients.

Seahorse Analyzer

In addition to this high-resolution oxygraph, the development of the high-throughput Seahorse extracellular flux analyzer (Agilent) has been crucial to the characterization of cancer cells metabolism [81]. The Seahorse system permits the simultaneous assessment of glycolytic and oxidative metabolism [82]. The Analyzer measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) at intervals of minutes. OCR, reported in pmol/minute, is an indicator of mitochondrial respiration, and ECAR, reported in mpH/minute, is largely the result of glycolysis. Nutrients and specific drugs, that have been preloaded into the drug delivery ports of the assay cartridge, are then pneumatically injected, sequentially, into the media in each well. After gentle mixing, OCR and ECAR measurements are made multiple times.

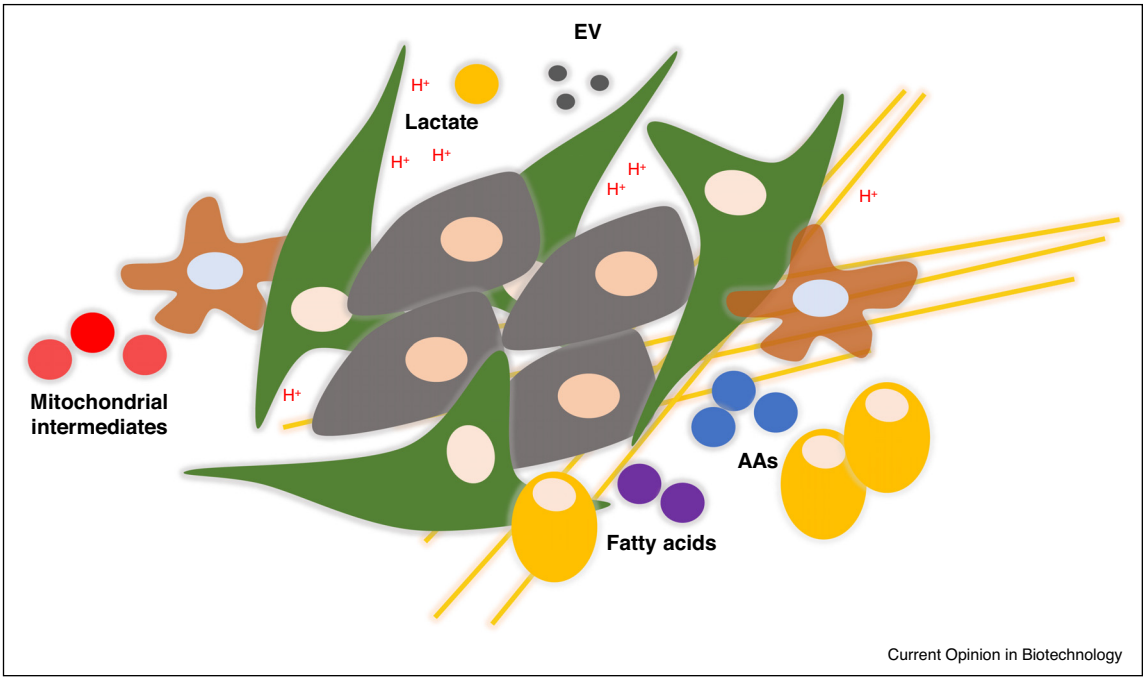
Some of these techniques, particularly metabolic ^{13}C-labeling, high-resolution oxygraphy and seahorse analyzer, can be applied in cellular metabolic nutrients exchange studies after MS exometabolomic characterization of molecules present in *in vitro* multicellular coculture or in TME itself. For example, using a specific ^{13}C-labeled nutrient it is possible to follow its metabolic fate in recipient cells, while through seahorse analyzer and high-resolution oxygraphy it is conceivable to determine the cellular phenotypic effect of such metabolites. Overall, these techniques provide a powerful platform to study the nutrient exchange between cellular components of TME to reach a better knowledge of cancer progression.

Computational approaches in metabolic cell communication

In recent years an increasingly acknowledged approach to study cancer metabolism is to simulate it through computational models. The classic approach to metabolic simulation is the application of ordinary differential equations (ODE) to representative sets of enzyme reaction equations, given initial concentration of proteins/metabolites and enzyme parameters. This approach has largely been used e.g. to describe the favorable properties of Warburg effect for biomass production in cancer [83] to predict uptake/release of metabolites or oncometabolites [84] or to study effects and fates of drugs [85]. Although highly valuable and informative, this approach is essentially infeasible when the number of reactions increases (up to a genome-scale level) or models of specific reactions are not available.

Thanks to previous pioneer works in prokaryotes [86], a different and feasible approach termed constrain-based modelling, specifically Flux Balance Analysis (FBA), has been established [87]. FBA considers cells at the steady-state and, given the stoichiometric coefficients of metabolites in the reactions (the so-called stoichiometry matrix) and several constraints, it derives the distribution of fluxes of metabolites inside the cell using linear modelling on an objective function (classically biomass or ATP

Figure 1



Sharing of environmental nutrients underpins tumour metabolic plasticity. Stromal and tumour cells (grey cells) enrich the environment with several nutrients that can be shared and exploited for different purposes. Release and exchange of lactate (yellow circle), mitochondrial intermediates (red circles), fatty acids (violet circles), amino acids (blue circles) and extracellular vesicles (grey circles) promote a reciprocal metabolic reprogramming of the different cellular populations, resident or recruited in the tumour environment.

production). While defining models to be addressed by FBA it is easy to isolate enzymes and metabolites in specific compartments and connect different compartments through actual or ad-hoc transport reactions. This enables the application of FBA to model eukaryotic genome-scale metabolism. Currently, the most comprehensive genome-scale model for human, Recon3D, encompasses >3300 genes, >8000 metabolites and >12 000 biochemical and transport reactions distributed over nine cellular compartments [88••].

Besides the interesting theoretical properties of FBA applied to genome-scale reconstructions, the most interesting aspect of such an approach is the possibility to plug -omics data into the analysis. In fact, enzymatic or transport reactions are intimately associated to genes thanks to boolean relationships known as Gene-Protein-Reaction expressions (GPR), that allow to both list the proteins (genes) composing hetero-multimeric proteins and specify the involvement of protein isoforms. Several algorithms for transcriptomic and proteomic data integration have been developed [89,90] and used to create, for many cell type and tissues, context-specific model in which the internal metabolic machinery is adjusted to fit specific cell status [91,92]. In addition, metabolomics can be used to determine intake and export rate of nutrients and byproducts, respectively, imposing appropriate constrains to models [93].

Since microenvironment is known to modulate cell metabolic behavior, it is relevant to observe that constrain-based modelling can easily incorporate the metabolites that a cell can receive or extrude, and many modelling works have focused much on variation of consumption (i.e. glucose, glutamine) or secretion (i.e. lactic acid) of metabolites. A much more complex task is modelling variations in physio-chemical conditions, the most important being the pH. To this aim, constrain methods can be applied to introduce variations in the main model for specifically addressing e.g. pH-dependent variation in enzyme activity.

Several methodological efforts have been proposed to improve metabolic models, mainly by integrating them with other types of network (e.g. transport [94•], signaling [95], regulatory [96], and others) in order to improve flexibility and precision [97].

In the past, the problem of combining models of different cells/tissues was sparsely addressed to approach plant [98] and human pathophysiology [99,100]. The same approaches applied to cancer metabolism, especially in the context of microenvironment, are largely unexplored apart from contributions from Damiani et al. that propose a population level FBA (popFBA) [101], recently applied to single cell RNAseq data (scFBA) [102].

Modelling the exchange of metabolites among (different) cells inhabiting the same microenvironment have the potential to be of great benefit for studying cancer requirements to survive and proliferate. Such a model, built upon -omics experiments specifically aimed at capturing cell-cell relationships (i.e. obtained from co-cultures, transwell-coltures or medium communicating plates), would allow a better simulation and investigation of metabolite exchange between cells, possibly shedding new lights in the complex micro-environment of a tumour. Additionally, increasing evidence from single-cell RNA-sequencing indicates that metabolic and transcriptomic variability exists within a tumour mass, and this has been interpreted as a strategy to provide survival and metastatic advantages in adverse or even hostile environments [103].

Concluding remarks

Significant advances in analytical methods that support the investigation of metabolites associated with nutritional exchanges within TME have furthered the discovery and characterization of metabolic programs critical to tumorigenesis. The integration of metabolomics analysis with established omics platforms has uncovered the metabolic rewiring seen in tumours, revealing higher metabolic plasticity/heterogeneity of tumour cells as well as metabolic vulnerabilities. Optimistically, an improvement in single-cell omics techniques [104••] and the refinement of new dynamic cancer-on-a-chip microfluidic systems, miniaturizing the biophisycal and metabolic features of a tumour niche [105], will provide higher resolution and accuracy in the characterization of metabolic heterogeneity deriving from the malignant and non-malignant cell subpopulations within TME, upon combination with analytical platforms such as MS. However, despite these technical developments, further refinement is required to fully quantify the cellular metabolism in real-time. The recent development of ex vivo metabolite tracing will likely drive future discovery and enhance therapeutic development.'

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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