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Metabolomics in rheumatic diseases: desperately seeking biomarkers

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Abstract

Metabolomics enables the profiling of large numbers of small molecules in cells, tissues and biological fluids. These molecules, which include amino acids, carbohydrates, lipids, nucleotides and their metabolites, can be detected quantitatively. Metabolomic methods, often focused on the information-rich analytical techniques of NMR spectroscopy and mass spectrometry, have potential for early diagnosis, monitoring therapy and defining disease pathogenesis in many therapeutic areas, including rheumatic diseases. By performing global metabolite profiling, also known as untargeted metabolomics, new discoveries linking cellular pathways to biological mechanisms are being revealed and are shaping our understanding of cell biology, physiology and medicine. These pathways can potentially be targeted to diagnose and treat patients with immune-mediated diseases.

Endogenous and exogenous low-molecular-weight molecules (<1–1.5 kDa) in a biological sample are generally referred to as metabolites. For decades, the identification and quantification of metabolites was primarily determined by analytical chemists and biochemists using targeted analysis of specific subsets of compounds[1–3]. This metabolic profiling, or metabolite target analysis (BOX 1), revealed the chemical nature of important compounds including vitamins, cofactors and amino acids. With the advent of systems biology and the ‘omics’ revolution, metabolomics[4,5] has enabled the rapid, simultaneous measurement of thousands of metabolites from minimal amounts of sample. Metabolic datasets obtained using both metabolomics and stable-isotope-assisted metabolomics[6–9] have been used to feed into different mathematical modelling approaches, including metabolic flux analysis (see Supplementary information S1 (figure)).

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Author contributions

M.G. and S.T. researched data for the article. All authors contributed to discussion of content, writing the article and reviewing and editing the manuscript before submission.

Competing interests statement

The authors declare no competing interests.

SUPPLEMENTARY INFORMATION

See online article: S1 (figure) | S2 (table)

Genes are subject to epigenetic regulation, and proteins to post-translational modification, but metabolites are direct signatures of biochemical activity, and might be easier to correlate with phenotypes. The response of a biological system to disease status or therapeutic intervention can be reflected in specific patterns of metabolites. To ensure the inclusion of all relevant molecules, metabolomic approaches should ideally be untargeted, hypothesis-free, comprehensive analyses of metabolites followed by advanced data processing[5]. However, owing to the computational complexity of untargeted analyses, targeted studies are also conducted.

The metabolome is the complete set of metabolites within a biological sample. Metabolic pathways can interact and overlap (FIG. 1 and the Metabolic pathways — reference pathway). In samples from the human body, ~3,000–5,000 metabolites have been detected, and many have already been identified. The metabolomes of biofluid and tissue samples are affected not only by genetics, but also by lifestyle factors including diet, drugs, exercise, gut microbiota, health-to-disease status, hormonal homeostasis and age. Metabolites can be endogenous, including lipids, small peptides, amino acids, organic acids, vitamins, carbohydrates, thiols and nucleic acids (see Supplementary information S2 (table)) or exogenous, such as drugs, environmental contaminants, food additives, toxins and other xenobiotics. Lipids, amino acids and carbohydrates are the most abundant metabolites in plasma[10].

Abundant endogenous metabolites

Lipids

Lipid metabolism, including uptake, transport, synthesis and degradation, is a complex process regulated by a number of signalling pathways. Lipids have important biological functions in energy storage, as structural components of cell membranes and as signalling molecules. The lipid components of biological membranes were originally considered to be merely structural, but considerable evidence now exists demonstrating the importance of lipid signalling via a variety of receptors[11] (TABLE 1). Alteration of lipid metabolism leads to changes in membrane composition and permeability, gene expression and protein distribution and function, as well as in cellular functions such as cell growth, proliferation, differentiation, survival, apoptosis, inflammation and motility, causing the development and progression of many diseases.

Carbohydrates

Carbohydrates have crucial roles in metabolism and signalling (TABLE 1). The monosaccharide glucose is an important energy source, and increases in glucose uptake and glycolysis have been associated with increased cellular proliferation by generating ATP and providing substrates for the synthesis of proteins, nucleic acids and lipids. Carbon compounds produced during glycolysis can also be diverted into either the oxidative or the nonoxidative branches of the pentose-phosphate pathway (PPP) for nucleotide and cofactor biosynthesis and redox balance. Indeed, NADPH, the main reducing cofactor in the cytoplasm, is required to quench reactive oxygen species during cellular proliferation.

Amino acids

Amino acids are nutrients and substrates for macromolecular synthesis. For example, glutamine, although not an essential amino acid, is well studied and highly abundant (~0.5 mM in blood). During cellular proliferation, glutamine provides nitrogen and carbon for the *de novo* synthesis of amino acids and synthesis of nucleotides through the hexosamine pathway, and can (particularly in hypoxic conditions associated with cancer) contribute to the generation of cytosolic acetyl coenzyme A, an intermediate in lipid biosynthesis[12] (Metabolic pathways — reference pathway).

Bioactivity and metabolite crosstalk

Metabolites can participate in intercellular communication[13] (TABLE 1). Metabolite exchange between stromal and parenchymal cells is common and essential for the function of numerous tissues. In both cancer and inflammation, metabolism and cellular communication are altered. Lactate from tumour cells, for instance, promotes tumour angiogenesis via activation of the hypoxia-inducible transcription factor HIF-1α[14]. Metabolic enzymes can also affect post-translational modification[15].

Metabolites also have signalling roles in immune cells, such as the induction of IL-1β expression by succinate via activation of HIF-1α[16]. Succinate and other metabolites such as acetyl coenzyme A are also involved in alteration of the epigenome[17,18]. Amino acids have been shown to affect cell signalling[19], recruitment[20] and proliferation of immune cells,[21] and members of several different categories of lipids have been identified as signalling molecules and cellular messengers[11].

Soluble inositol polyphosphates and membrane polyphosphoinositide lipids[22], have numerous functions. Phosphatidylinositol (3,4,5)-trisphosphate, for example, modulates cell growth, proliferation and motility[11]. Ceramides and sphingosines have proapoptotic and antiproliferative actions[23], but phosphorylation of sphingosine converts it to sphingosine-1-phosphate, which promotes cell growth and proliferation[24]. Eicosanoids[25] and lysophosphatidic acid[11] also have autocrine and paracrine actions through binding to a family of G-protein-coupled receptors.

Metabolomics databases

Metabolomes vary considerably between tissues and biofluids, and databases have been developed for the large amounts of information generated in studies of different systems[26]. One of the most highly utilized is the Human Metabolome Database or HMDB[27], an open-access resource containing detailed information on >40,000 metabolites. Other resources with information relating to compounds or spectroscopy are the Kyoto Encyclopedia of Genes and Genomes (KEGG)[28], LipidMaps[29], PubChem[30], Chemical Entities of Biological Interest (ChEBI)[31], Madison Metabolomics Consortium Database (MMCD)[32], METLIN[33] and MassBank[34] databases. The Metabolomics Standards Initiative (MSI)[35] was created to help to avoid discrepancies between databases and to promote consistency and reproducibility between laboratories with different instrumentation and methods of analysis.

State-of-the-art technologies

Profiling the metabolome is challenging, owing to the intrinsic heterogeneity of the compounds involved[36–38], and it becomes even more problematic when absolute quantification of one or more biomarkers is required, particularly in the context of clinical studies[39]. Before performing any metabolite analyses, careful design of sample collection, data processing and data analysis is crucial to limit unwanted bias. Adding quality control during data acquisition is important to obtain reproducible results and ensure generation of meaningful data: if samples are not collected properly, or stored and processed uniformly, the data generated could be invalid. Sample collection, storage, and processing procedures are extremely important for the conduction of successful metabolomic studies, which also require careful design to minimize and account for the effects of factors such as gender, age, diet, fasting state, exercise and physical activity, the use of drugs, medications and other active substances, and the time of day of sample collection.

Metabolomic analysis can involve different analytical platforms. These methodologies include ultraviolet spectroscopy, Fourier transform infrared spectroscopy and Raman spectroscopy[40], but the most commonly utilized platforms are NMR spectroscopy and mass spectrometry (MS), both of which have advantages and disadvantages (BOX 2).

NMR spectroscopy

NMR spectroscopy, although having lower sensitivity than MS, is a robust metabolomic platform with several advantages. NMR is currently the best technique for chemical structure elucidation[41]; it requires only minimal sample preparation, and is nondestructive, inherently untargeted, highly reproducible[42,43] and intrinsically quantitative[44–46]. An advantage of NMR spectroscopy is the ability to bridge the gap between *in vitro*, *ex vivo* and *in vivo* studies, with the use of high-resolution magic-angle spinning to profile intact tissues and magnetic resonance spectroscopic imaging for *in vivo* applications[47]. Developments in the sensitivity and resolution of NMR instrumentation come about through improvements in the availability of superconducting materials, the strength of magnetic fields[48] and cryogenic probe technology[49].

Historically, NMR probes required large sample sizes (500–600 μl), but advances in microprobe development now enable the analysis of samples ≤30 μl[50]. Cooling microprobe coils with a stream of helium gas (20–30 K)[51] enables 60–80-fold increases in the NMR signal-to-noise ratio compared with conventional 5 mm room-temperature probes.

In the metabolomic profiling of biological samples, the majority of NMR spectra are acquired by 1D NMR for the high throughput required for screening purposes[52]. However, compared with 1D NMR, ultrafast and nonuniform sampling has shown potential to reduce signal overlap and acquisition time and to enhance signal sensitivity by increasing the dimensionality of NMR spectra[53–55]. In a study of the human urine metabolome[56], high-esolution NMR detected and quantified >200 metabolites in an untargeted fashion, including >100 metabolites that were identified by NMR but not by other techniques. However, the need to increase the number of detected metabolites to obtain a more comprehensive

description of the metabolic status has motivated researchers to add — or to switch to — MS-based analysis[56–61].

Mass spectrometry

Currently available mass analysers[62] include triple quadrupole, quadrupole time-of-flight (TOF) and linear ion trap–Fourier transform ion cyclotron resonance, as well as orbitrap devices[63,64]. Metabolites can either be directly injected into the mass spectrometer as a mixture, or they can first be separated, by one of a variety of methods. Direct injection without separation into a high-mass-accuracy mass spectrometer (such as an orbitrap) is commonly called direct infusion MS[65] or shotgun MS[66,67]. This high-throughput, untargeted and comprehensive technique is ideal for a first metabolic screen, but the lack of initial separation means that metabolites with the same mass will overlap, interfering with identification.

Gas-chromatography–MS (GC–MS) and liquid-chromatography–MS (LC–MS) are the most common MS-based metabolomics methodologies. GC–MS is ideal for the analysis of volatile compounds, and is also commonly used for targeted metabolic analysis of organic acids, amino acids, sugars and fatty acids, following chemical derivatization. GC–MS is one of the most robust MS-based techniques for metabolomic[68] and lipidomic quantification[69], but GC–MS systems commonly have low-resolution spectrometers, and high-resolution LC–MS platforms are increasingly being utilized for both untargeted and targeted metabolic analyses. In LC–MS, in addition to choosing between MS systems, the choice of liquid chromatography column (regardless of the use of high pressure or ultrahigh pressure) is vital to the success of the profiling experiment, and in general multiple columns are necessary for optimal detection of metabolites[70]. LC–MS systems can detect thousands of features and identify hundreds of compounds[71]. However, many features cannot be identified using available databases. Tandem MS (MS/MS)[27,29,33] can aid in identification; alternatively, spiking a sample with a chemical standard can help to identify a putative metabolite[72].

Stable-isotope-assisted metabolomics

Identification and quantification of metabolic biomarkers can provide a metabolic snapshot of the status of a living organism, but cannot provide an unambiguous picture of the metabolic flux between cellular compartments. For instance, an increase in the concentration of a metabolite can be associated with either the upregulation of the enzyme that synthesizes the metabolite or the downregulation of the one that consumes it. Stable-isotope labelling (with an isotope such as ^{13}C) of a precursor (such as glucose or glutamine) enables tracking of its cellular fate *in vitro* or *in vivo*[53,73]. Datasets from such experiments can be fed into mathematical models such as metabolic flux analysis[6–9,74,75] (see Supplementary information S1 (figure)).

Metabolomic studies in rheumatic disease

Metabolomics can provide important information relating to pathogenesis and disease activity in rheumatic conditions. The fundamental rationale in metabolomics is that perturbations in a biological system caused by disease will lead to correlated changes in the

concentrations of certain metabolites. Although in some situations (such as genetic metabolic disease) the identification of a single, robust diagnostic metabolite migth be possible, in many others (including rheumatic disorders) the perturbations involve the activation of multiple pathways. NMR and MS can identify biomarkers and patterns of change that are highly discriminatory for these perturbations and for disease states. Disease-specific metabolic pathways can indicate potential therapeutic targets, to enable alteration of the metabolic activity implicated in pathogenesis. They can also identify disease-specific biomarkers.

Biomarkers are anatomical, physiological, biochemical or molecular variables or imaging features that enable diagnosis and prognosis of disease and evaluation of the effects of treatment. The accessibility of biomarkers is important, and blood and urine are more accessible than synovial tissues or fluids in joints affected by rheumatic diseases. Despite the relative inaccessibility, metabolic profiling using intact tissue has gained momentum as an approach for understanding the molecular basis of disease.

The study of metabolomics in inflammatory disease represents a new approach to identifying biomarkers beyond autoantibody profiling and transcriptomics. Several metabolomics studies have focused on the identification of metabolites associated with rheumatic diseases or the prediction of response to treatment. In general, the results of these studies have been promising, but most included small numbers of patients, with heterogeneous clinical characteristics and treatments, and require confirmation[76]. Studies to identify correlations between metabolomic profiles and inflammation, bone and cartilage damage and the effects of therapeutic intervention are needed. The inclusion of normal controls matched for age and sex, and of populations affected by other arthritic diseases, as well as validation cohorts will help to determine whether metabolic signatures can distinguish between subsets of patients and help in diagnosis and prognosis.

The metabolomics of rheumatic disease have been studied in humans and in murine models. Some elements of metabolism are similar in different species, and biomarker discovery might benefit from the use of targeted animal models in which induction of disease and treatment can be controlled, and correlations between the metabolomes of serum and joints can be evaluated.

Systemic lupus erythematosus (SLE)

Alteration of the metabolic profile in SLE has been studied by ^{1}H-NMR on serum samples obtained from patients with SLE ($n = 64$), patients with rheumatoid arthritis (RA; $n = 30$) and healthy controls ($n = 35$)[77]. In patients with SLE, significant reductions in the levels of valine, tyrosine, phenylalanine, lysine, isoleucine, histidine, glutamine, alanine, citrate, creatinine, creatine, pyruvate, cholesterol, glycerol and formate were detected in comparison with the control population. Apart from the decrease in creatine, the same profile was detected in patients with RA as in those with SLE, suggesting a correlation of these metabolic changes with inflammation.

A comparison of sera from 20 patients with SLE and nine healthy controls using LC–MS and GC–MS platforms identified >100 metabolites that were significantly different in the

SLE population[78]. Validation of these findings was performed with an independent cohort of 38 patients with SLE, along with 20 patients with RA and 14 healthy controls[78]. Compared with the controls, sera from patients with SLE showed evidence of profoundly dampened glycolysis, Krebs cycle, fatty acid β-oxidation and amino acid metabolism, suggesting reduced energy biogenesis from all sources[78]. Whereas levels of long-chain fatty acids were significantly reduced, those of medium-chain fatty acids were elevated in association with SLE. The metabolomes in patients with SLE exhibited profound lipid peroxidation reflective of oxidative damage, with deficiencies in the cellular antioxidant glutathione, as well as methyl group donors required for glutathione regeneration[78].

Urinary metabolites that discriminate between proliferative and pure membranous lupus nephritis have been identified with NMR spectroscopy in a pilot study involving seven patients[79]. Urinary citrate levels were eightfold lower in patients with class V lupus nephritis than in those with class III or class IV disease (P <0.05), in whom the levels were normal. Conversely, levels of urinary taurine were mostly normal in patients with class V disease, but >10-fold lower in those with class III or class IV disease, suggesting that these urinary metabolites can serve as biomarkers to help discriminate between different classes of lupus nephritis.

Preclinical models can provide important clues to help refine searches for biomarkers in human disease. Rapid-resolution liquid chromatography coupled with quadrupole TOF-MS (RRLC–Q-TOF-MS) has been utilized to acquire metabolic profiles of serum samples obtained from mice treated with single intraperitoneal injections of pristane, which induces an SLE-like disease characterized by the production of autoantibodies[80]. These mice had altered levels of 13 metabolites associated with the metabolism of unsaturated fatty acids (UFAs), phospholipids and tryptamine[80].

The results of these metabolomics studies suggest that an imbalance in lipid profiles might contribute to disease, especially in relation to arachidonic acid metabolism. Evidence derived from studies involving both human and murine[80] samples implicates disorder of UFA metabolism in SLE and SLE-like disease. UFAs have an important role in the maintenance of normal physiological functions, including regulation of the immune response. Among the UFA metabolites that are differentially regulated in SLE, 12-hydroxyeicosatetraenoic acid (12[S]-HETE) is an active metabolite of arachidonic acid that is produced through the arachidonate 12-lipoxygenase pathway. This metabolite has a prominent role in the promotion of inflammation, causes the accumulation of extracellular matrix and induction of mesangial-cell hypertrophy, and is involved in the pathogenesis of diabetic nephropathy[81]. An increase in the level of 12(S)-HETE in SLE might promote kidney inflammation and cell hypertrophy, thereby aggravating the symptoms of kidney disease. Other metabolites, such as 5-hydroxyicosatetraenoic acid and leukotriene B4, are also elevated in SLE and can contribute to the pro-inflammatory milieu and prothrombotic state.

Osteoarthritis (OA)

The alteration of the metabolic profile in OA has been studied in media from the culture of synovial explants dissected from diseased joints in patients with no OA or early OA

compared with end-stage OA (11 patients per group)[82]. The samples were subjected to global metabolic profiling with LC–MS and GC–MS, resulting in the identification of 11 compounds with significantly different concentrations in the two groups[82]. The metabolite profiles suggested that abnormal degradation of collagen contributes to an 'OA signature' in the blood. However, given the discrepancy in the mean ages of the patients (67 years in the end-stage OA group versus 18 years in the group with no OA or early OA), some of the observed metabolite changes could be secondary to age-related chondrocyte changes, rather than being specific to OA[82].

A study in which OA phenotypes were classified by the results of metabolomic analyses was limited by the absence of synovial fluid samples from healthy people, and by the failure to associate the phenotypes with clinical data[83]. In another study, synovial fluid samples were collected from 55 patients with symptomatic chronic knee OA and 12 normal human cadaveric knee joints; this study also included a validation group and was age-matched[84]. The samples were analysed by ^{1}H-NMR and GC–MS, and the differences in metabolic profiles were indicative of hypoxic conditions in diseased and inflamed knee joints, and high energy requirements in patients with OA[84]. Similar alterations have been described previously[84–88], with some variations in the involvement of specific metabolites, possibly owing to the use of animal models[84–86,88] or the lack of a healthy human control group[87]. These studies did not include samples from other inflammatory arthritides, so further studies are needed to determine the specific metabolomic profile of OA.

Studies have also been conducted with serum or urine samples to determine the metabolomic signature of OA. In a study with MS/MS analysis of serum samples from patients with knee OA, the ratio of branched-chain amino acids (valine and leucine) to histidine was described as a novel biomarker[89]. Analysis by ^{1}H-NMR of urine samples from 47 controls and 45 individuals with OA identified a metabolite profile suggestive of altered energy metabolism that was strongly associated with OA[90]. A comparison of plasma and synovial fluid from patients with primary knee OA (with metabolic profiling by MS/MS)[91] found modest correlation between metabolite concentrations, but higher correlation between metabolite ratios, which are considered proxies for enzymatic reaction rates. Assessment of metabolite ratios should be considered when using plasma as a surrogate for synovial fluid in biomarker identification in OA and probably in other diseases.

RA and inflammatory arthritides

Metabolic profiling has been explored in synovial fluid in inflammatory arthritides. Synovial fluid samples from 38 patients with RA, ankylosing spondylitis, Behçet disease or gout were analysed by GC–MS[92], and 20 metabolites were selected as potential biomarkers to discriminate RA from the other conditions. Higher abundance of a number of metabolites in samples from patients with RA indicated activation of energy pathways such as the tricarboxylic acid (TCA) cycle, as well as amino-acid metabolism[92]. However, the levels of inflammatory markers were not measured, so these changes could be related to a greater degree of inflammation in RA than in the other conditions.

Untargeted screening of lipids in synovial fluid samples from patients with RA has been performed with an LC–MS/MS screening platform[93]. Approximately 70 different

components from distinct lipid classes were detected, although no control group was analysed[93]. A targeted lipidomics strategy was developed for quantification of anti-inflammatory bioactive fatty acids maresin 1, lipoxin A4 and resolvin D5. In a study of synovial fluid samples from unaffected controls (n = 9) and from patients with early OA (n = 17), late OA (n = 13) or RA (n = 30), phospholipid species were quantified by MS/MS[94]. Several lipid classes were identified, and the highest concentrations (with the exception of phosphatidylserine) were observed in late OA[94]. Samples from patients with RA also had higher concentrations of these lipids than control samples. These observations suggest that phospholipid changes are secondary to cartilage damage; their production could protect cartilage from friction-induced mechanical damage. However, significant differences in age and gender between groups made it difficult to conclude that these differences were disease-specific[94].

Studies have also been carried out to identify metabolites in serum and plasma that can help in diagnosis, prognosis and the prediction and measurement of response to treatment in rheumatic diseases. In a comparison of serum metabolic signatures in OA, RA, ankylosing spondylitis and gout by MS[95], homoserine, 4,8-dimethylnonanoyl carnitine, glyceraldehyde, lactic acid, dihydroxyfumaric acid and aspartic acid were identified as candidate markers in the four types of arthritis, compared with healthy controls. In another study, with ^{1}H-NMR characterization of serum samples from patients with established RA, early arthritis and healthy controls, levels of several metabolites, such as 3-hydroxybutyrate, glucose, lactate, and urea, were correlated with the extent of inflammation[96].

The data from these studies suggest that upregulation of glycolysis, the TCA cycle, metabolism of glycine and serine, fatty acid and amino acid metabolism occurs in all these inflammatory conditions, indicating that these pathways might not be suitable sources of disease-specific biomarkers. Further studies are required to complement these investigations and others involving RA and inflammatory arthritides (such as psoriatic arthritis and ankylosing spondylitis) to identify specific biomarkers for each condition[97–101].

In a preclinical study[102], a serum metabolomic method involving RRLC–Q-TOF-MS was performed for an evaluation of the metabolic changes in collagen-induced arthritis (CIA) in rats. Herein, 10 metabolites (suggestive of dysregulation of the arachidonic acid and phospholipid metabolic networks) were found to be associated with the pathogenesis of CIA. The relationships between eicosanoid metabolism, arthritis and inflammation have been reviewed previously[25].

Attempts to correlate metabolite profiles and response-to-treatment have had promising results. In one study[103], a ^{1}H-NMR metabolomic approach was applied to 38 patients with active RA. After 24 weeks, patients who responded to methotrexate treatment showed significant elevations in serum levels of uric acid, taurine, methionine, glycine, histidine and hypoxanthine, and reductions in levels of uracil, TMAO, α-oxoglutarate, aspartate, and tryptophan, compared with patients in whom methotrexate was not effective[103]. NMR analysis of urine from 16 patients with RA and 20 with psoriasis (before anti-TNF therapy)[104] identified upregulation of histamine, glutamine, phenylacetic acid, xanthine, xanthurenic acid and creatinine, and downregulation of ethanolamine, p-

hydroxyphenylpyruvic acid and phosphocreatine in samples from patients who had a good response to therapy.

Disease-specific metabolic pathways

Because metabolic pathways are so closely related, the alteration of one of these pathways will probably have wider effects on the metabolome. Analysis of metabolomics data might identify specific metabolic pathways and activated signalling pathways associated with the pathogenesis of a disease. Metabolic changes described in specific cell types or bioactive metabolites involved in the pathogenesis of the disease[105] might also correlate with changes observed in metabolomics studies.

Many signalling pathways activated under inflammatory conditions have profound effects on cellular metabolism, supporting cell growth and survival. These adaptations must be implemented in the stressful and dynamic microenvironment of inflamed tissues, where concentrations of crucial nutrients such as glucose, glutamine and oxygen are spatially and temporally heterogeneous. Therefore, multiple molecular mechanisms converge to alter core cellular metabolism and provide support for three basic needs of dividing cells: ATP generation to maintain energy status; biosynthesis of macromolecules; and tight maintenance of appropriate cellular redox status. Cells acquire alterations to the metabolism of the major classes of macromolecules — carbohydrates, proteins, lipids and nucleic acids. The study of cancer cell metabolism has successfully identified cancer-specific metabolic changes, but other cell types, such as lymphocytes and stromal cells, are also subject to major metabolic challenges upon activation, and adopt specific metabolic programmes to adapt to changing environmental conditions.

Immune cells have specific metabolic signatures related to effector function. Metabolic changes occurring in T cells[106–110], macrophages[111] and dendritic cells[112,113] have been extensively reviewed, but less information is available with regard to stromal cells, including fibroblasts, and endothelial cells[105]. Emerging evidence also indicates that metabolism is not only involved in the determination of cell differentiation and function, but also that metabolic changes contribute to the pathogenesis of cancer, diabetes and inflammatory diseases[105]. Some of these changes are summarized in FIG. 2 and BOX 3.

SLE

Glycolysis and mitochondrial oxidative metabolism are elevated in $CD4^+$ T cells from lupus-prone mice (compared with nonautoimmune controls) and also in $CD4^+$ T cells from patients with SLE; the elevation correlates with the patients' activation status, and IFN-γ production is significantly reduced by metformin[114]. Treatment with a combination of metformin and 2-deoxy-D-glucose normalizes T-cell metabolism and reverses changes in the levels of disease biomarkers in animal models of lupus, suggesting a promising therapeutic approach for SLE[114]. However, serum metabolomics in patients with SLE have shown reduced glycolysis and Krebs cycle activity relative to healthy controls, and the contribution of these pathways to SLE pathogenesis is yet to be determined[78].

$CD4^+$ T cells from patients with SLE display an altered profile of lipid-raft–associated glycosphingolipids compared with those from healthy controls[115]. Glycosphingolipids are essential for many cellular processes and are enriched predominantly in lipid rafts, influencing a range of T-cell functions including T cell receptor-mediated signalling and apoptosis, as well as recycling and endocytosis of membrane-associated receptors and signalling molecules. Inhibition of glycosphingolipid biosynthesis *in vitro* normalizes glycosphingolipid metabolism, corrects $CD4^+$ T-cell signalling and functional defects, and decreases anti-double stranded DNA (dsDNA) antibody production by autologous B cells in patients with SLE[115]. In metabolomics studies, no changes in this subset of lipids have been detected in blood, although further *in vivo* and *in vitro* studies are needed to determine if these particular T-cell metabolic changes can be detected.

RA

The observation of increases in serum lactate in metabolomics studies conducted in the setting of inflammatory arthritides, including RA, suggests an alteration of glycolysis. However, the metabolic status of naive $CD4^+$ T cells has been examined in patients with RA, thereby excluding T cells that are directly involved in the inflammatory process itself. These naive RA T cells did not produce as much ATP and lactate as naive T cells from healthy, age-matched controls, although they proliferated vigorously[116]. Molecular analysis of the underlying defect responsible for this metabolic alteration identified 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) as a rate-limiting enzyme in the glycolytic pathway that was significantly decreased in T cells from patients with RA, compared with healthy controls. Fibroblast-like synoviocytes (FLSs) from patients with RA also have a higher baseline glycolytic rate than FLSs from patients with OA[117,118]. Expression of GLUT-1 (solute carrier family 2, facilitated glucose transporter member 1) also correlated with migration and expression of metalloproteinases. Notably, glucose deprivation or treatment with glycolytic inhibitors such as 2-deoxy-D-glucose and bromopyruvate impaired cytokine secretion, proliferation and migration in FLSs, and glycolytic inhibition by bromopyruvate administered *in vivo* in a serum-transfer animal model significantly decreased arthritis severity compared with untreated animals[118]. Thus, the increase in lactate detected in metabolomics studies[95,96] could be secondary to the increase in glycolysis in FLSs.

The choline pathway is highly active in FLSs[119]. Choline kinase-α (CK), the enzyme that catalyses the first step in the cytidine-diphosphate–choline pathway, is essential for phosphatidylcholine production. In comparison with healthy tissue, elevated levels of CK have been associated with malignant transformation, invasion and metastasis in some human cancers[120–124]. CK is expressed in synovial tissue in RA and in cultured FLSs; furthermore, a CK inhibitor suppresses the aggressive properties of cultured RA FLSs, including cell migration and resistance to apoptosis[119]. In a serum-transfer model of arthritis, pharmacological CK inhibition (compared with vehicle-only) prevented development of arthritis when administered before serum transfer, and significantly decreased levels of markers of arthritis in treatment of established disease[119]. Notably, in metabolomics studies, choline levels have been correlated with the degree of inflammation in RA, and several lipid alterations have been identified in RA samples[93,104].

Sphingosine kinase 1 (SPK-1) phosphorylates sphingosine to make sphingosine-1-phosphate (S1P), a bioactive lipid involved in the pathogenesis of several autoimmune diseases[24]. SPK-1 blockade suppresses the release of cytokines and MMP-9 in peripheral blood mononuclear cells from patients with RA. In addition, downregulation of SPK-1 — either by an siRNA approach or in transgenic mice expressing human TNF and deficient in SPK-1 — limits synovial inflammation and joint pathology[125,126]. The synovia and synovial fluids of patients with RA exhibit significantly higher levels of S1P than those of patients with OA[127].

Activated FLSs from arthritic human patients and animal models express autotaxin, which catalyses the conversion of lysophosphatidylcholine to lysophosphatidic acid[128–130]. This expression is induced by TNF, which also acts synergistically with lysophosphatidic acid to induce fibroblast activation and effector functions[130]. Conditional genetic ablation of autotaxin expression in mesenchymal cells, including FLSs, results in disease attenuation in animal models of arthritis. Notably, high levels of lysophosphatidylcholine and low ratios of phosphatidylcholine to lysophosphatidylcholine in plasma represent a reliable measure of inflammation[131].

Metabolic targeting in rheumatic diseases

The metabolic rewiring of cancer cells and immune cells has been viewed as a promising source of drug targets[132–138]. Resetting the altered metabolomes in these diseases, either by targeting selected molecules or by supplementing the diet with essential metabolites, such as fatty acids, offers novel opportunities for disease modulation. Several different approaches have been explored, leading to the identification of agents that are now close to entering clinical evaluation. Few metabolic inhibitors have been developed so far, reflecting the recent rediscovery of the field, as well as concerns regarding reproducibility of results and toxicity in cells undergoing intensive proliferation. Whether this approach will result in effective drugs for the treatment of rheumatic diseases, with effects beyond those of biological and kinase-inhibitor therapies, remains to be determined.

Rheumatologists already use the metabolic inhibitors methotrexate and leflunomide for the treatment of inflammatory arthritis[139]. Teriflunomide, the active metabolite of leflunomide, inhibits the mitochondrial enzyme dihydroorotate dehydrogenase. This enzyme is involved in *de novo* synthesis of pyrimidines, including uridine monophosphate (UMP), which is required for the synthesis of DNA and RNA; thus, leflunomide inhibits the reproduction of rapidly dividing cells, especially lymphocytes. Methotrexate, developed as a folic acid analogue, inhibits purine and pyrimidine synthesis, which accounts for its efficacy in the treatment of cancer, as well as for some of its toxicity. Other consequences of methotrexate treatment, such as adenosine accumulation, might also contribute to its disease-modifying effect in RA.

Some drugs that target altered metabolism in cancer could also have therapeutic value in rheumatic disease. Attempts to block aerobic glycolysis in tumour cells have not yet been effective[140,141]. Therapeutic approaches targeting numerous points in the glycolytic process are currently being evaluated, including inhibition of lactate dehydrogenase[142] and

inactivation of the glucose-uptake enzyme GLUT-1 (REF. 143) and monocarboxylate transporters[144], which are responsible for conveying lactate across the plasma membrane. Interest is also being refocused on interventions that preferentially target the inducible hexokinase-2 isoform rather than the ubiquitous and constitutively expressed hexokinase-1 (REFS 145–147). Hexokinases control the first committed step of glucose metabolism. Expression of hexokinase-2 is restricted in normal adult tissues, making it an attractive target for selective inhibition, which could be safer than global regulation of glycolysis.

Ligand binding to G-protein-coupled receptors induces dissociation of heterotrimeric G proteins, releasing βγ subunits that activate phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit-γ isoform (PI3Kγ) to produce large, transient elevations in levels of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3)[22,148]. The lipid kinase PI3Kγ integrates multiple signals from chemokines, complement fragments, formylated bacterial peptides and other stimuli, and is essential for chemokine-induced leukocyte migration *in vivo*. PI3Kγ is the target of considerable pharmacological efforts to treat inflammatory disease[149]. Inhibitors of PI3Kγ have shown therapeutic effects in mouse models of RA and SLE, attenuating the migration of neutrophils and lymphocytes, respectively, into inflamed tissue[149].

The sphingosine kinase pathway is another target[24]. Fingolimod, when phosphorylated by SPK-1, functions as an S1P-receptor agonist and targets four of the five known S1P receptors[24]. Fingolimod interferes with the exit of lymphocytes from lymph nodes — attenuating autoimmune disease such as multiple sclerosis — and is under consideration for treatment of other inflammatory and allergic conditions[150].

As high proliferation rates entail a considerable demand for the generation of novel phospholipid bilayers, targeting *de novo* lipogenesis[151] or glutaminolysis[152] also constitutes a rational approach for metabolic targeting. Several enzymes involved in these molecular circuitries, including fatty acid synthase[153], ATP citrate lyase[154], acetyl-coenzyme A carboxylases[155], choline kinase[156,157], monoglyceride lipase[158] and 3-hydroxy-3-methylglutaryl coenzyme A reductase[159], have been ascribed critical roles in oncogenesis or tumour progression *in vivo*, and might be reasonable therapeutic targets in rheumatic disease.

In addition to assessing whether therapies ultimately cause changes in metabolic pathways, metabolomics analysis can also measure drug pharmacokinetics[160]. Pharmacometabolomics has the capability to monitor how patients respond metabolically to drugs, and to determine whether the metabolic response is correlated with inflammation, adverse events and response.

Future directions

The science of metabolomics in the context of disease is young, but has seen rapid and impressive progress. New technologies are now being developed to complement existing methods; for example, metabolomic genome-wide association studies (mGWAS) integrate multiple layers of molecular data[161–164]. Metabolomic data can be quantitative, and

mGWAS can uncover genetic variants that affect metabolite levels. Associations between single-nucleotide polymorphisms and specific metabolites indicate the biological mechanisms that underlie the genetic changes.

The influence of the microbiome on the metabolome is an area of increasing interest[165–167]. Perturbations of intestinal microbiota composition or function could have important roles in the development of diseases associated with altered metabolism, because intestinal microbiota can regulate the absorption, metabolism and storage of host metabolites. Dysbiosis of intestinal microbiota can directly perturb host immune regulatory networks. For example, butyrate, acetate and propionate — the main bacterially derived short-chain fatty acids (metabolites from bacterial fermentation of dietary fibre, highly enriched in the colon) — control differentiation and function of mucosal T_{REG} cells[168]. These relationships suggest that intestinal microbial function should be incorporated into an in-depth study of the prominent disorders of metabolism and the immune system in rheumatic diseases, and the possibility of treatment by regulation of the intestinal microbiota.

Conclusions

Metabolomics encompasses a powerful suite of technologies that enable the analysis of a wide range of small molecules that are involved in many aspects of physiology and pathology. Characterization of the differences in the metabolome between healthy and diseased states can improve our understanding of the mechanisms underlying pathological processes. As technology advances and our understanding of metabolic perturbations in rheumatic disease grows, new therapeutic targets and diagnostic tests will undoubtedly emerge. This process will require collaborations between clinicians, laboratory scientists and bioinformaticians. Ultimately, scientific advances must be translated into easily accessible, structured data that can be used by clinicians at the point-of-care for decision-making in the management of patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

- Along with other ‘omics’ approaches, metabolomics — the comprehensive analysis of all metabolites in a system — represents a change from the traditional analysis of single genes, transcripts, proteins or metabolites
- Improvements in analytical techniques and pattern-recognition methods have led to a rise in the numbers of untargeted and targeted metabolic studies that are being performed
- Understanding metabolic changes that are specifically associated with the pathogenesis of autoimmune diseases should lead to novel insights into disease mechanisms and to new strategies for treatment of rheumatic diseases
- The feasibility of metabolomics for biomarker discovery in rheumatology is supported by the assumption that metabolites are important players in biological systems and that diseases cause disruption of metabolic pathways

Box 1

Definitions in metabolomics

Metabolism

The sum total of all chemical reactions that occur in the cell. Metabolic processes are usually classified as catabolic, which usually release energy, and anabolic, which build new molecules and usually require energy.

Metabolites

Small biological compounds produced during metabolic activity, usually with a molecular mass below 1,500 Da. Typical metabolites include amino acids, carbohydrates, organic acids, lipids and nucleotides.

Metabolome

The entire set of metabolites present in a given compartment — such as a cell, tissue or body fluid — under a particular set of physiological conditions. The metabolome can include many heterogeneous molecules at a range of concentrations, including endogenous and exogenous metabolites. The cell metabolome can be divided into intracellular and extracellular metabolites, which constitute the endometabolome and exometabolome, respectively.

Metabolomics

A “comprehensive analysis in which all the metabolites of a biological system are identified and quantified” (REF. 4). As with other ‘omics’ techniques, metabolomics is an untargeted and comprehensive analysis.

Metabolic profiling (metabolite target analysis)

The identification and quantification of a predefined subset of metabolites associated with a specific metabolic pathway. Sometimes called targeted metabolomics[169], a misnomer as metabolomics implies an untargeted approach.

Metabolic fingerprinting

A rapid, high-throughput metabolite analysis of biological samples (cells, tissue, blood or urine) to determine the health status of an organism.

Metabolic footprinting

The analysis of metabolites excreted and secreted by prokaryotic and eukaryotic cells (the exometabolome).

Lipidomics

A subdivision of metabolomics, defined as “the full characterization of lipid molecular species and of their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation” (REF. 170).

Stable-isotope-assisted metabolomics

Metabolomic analysis following stable isotope incorporation, as in metabolic-flux-balance analysis using ^{13}C tracer.

Box 2

Pros and cons of current metabolomics technologies

Pros of gas-chromatography mass spectrometry (GC–MS)

- Detection of subpicomolar concentrations possible; can detect ≥200 metabolites per sample
- Good relative quantification
- Moderate mass accuracy (<50 ppm), improved by GC coupled to Orbitrap MS
- Minimal sample requirement
- Analysis of volatile compounds does not require derivatization
- Benchtop instrumentation
- Relatively inexpensive instrumentation (<USD$100,000)
- Moderate instrumentation warranty cost

Cons of GC–MS

- Injected sample cannot be recovered
- Derivatization or extensive sample preparation required for nonvolatile metabolites
- Derivatization procedures vary and can lead to formation of several products from a single metabolite
- Stereoisomers not identified

Pros of liquid-chromatography mass spectrometry (LC–MS)

- Detection of subfemtomolar concentrations possible; can detect ≥1,000 metabolites per sample
- Outstanding mass accuracy (<1–3 ppm)
- Detects wide size range of metabolites (≥50 Da)
- Minimal sample requirement
- Good relative quantification
- Increasing number of benchtop instruments becoming available
- Metabolite imaging possible with matrix-assisted laser desorption ionization (MALDI)

Cons of LC–MS

- Sample unrecoverable
- Molecular ionization susceptible to sample–matrix effects

- Requires isotopically enriched internal standards for absolute quantification
- Stereoisomers not identified
- Moderately expensive instrumentation (>$300,000–$1,000,000)

Pros of NMR

- Unbiased
- Nondestructive
- Definitive chemical structural identification
- Provides relative and absolute quantification
- Rapid acquisition of 1D ^{1}H spectra (1–5 min)
- Minimal sample preparation
- No chromatographic separation required
- Outstanding reproducibility (>98%)
- Metabolites detected in solution, semi-solid and solid samples
- Stereoisomers can be identified
- Can be applied *in vivo*

Disadvantages of NMR

- Nanomolar detection range of ~50–200 metabolites per sample
- Slow acquisition for 2D spectra (≥4 h)
- Majority of NMR probes require large sample volume (160–600 μl)
- High magnetic-field precludes benchtop instrumentation
- Expensive instrumentation (>$500,000–$2,000,000)

Box 3

Metabolic changes in activated cells

Activated[13,105,106,171] or malignant[172–175] cells shift from oxidative phosphorylation to glycolysis for ATP generation (FIG. 2). Although the glycolytic pathway is an inefficient way to produce ATP from glucose, the glycolytic intermediates can be used as precursors for nucleotide, amino acid, phospholipid and triglyceride biosynthesis. Nonmetabolic functions of glycolytic enzymes have been described[176]. Glycolytic products such as lactic acid[14] and succinate[17] can function as signalling molecules to control transcriptional responses.

The PPP is required for the synthesis of ribonucleotides and is a major source of NADPH[177], which is required for and consumed during fatty acid synthesis and the scavenging reactive oxygen species. The PPP enables glycolytic cells to meet their anabolic demands and combat oxidative stress. A glycolysis–PPP axis is also involved in M1 macrophage polarization[178].

Fatty acid metabolism is increased in tumour cells, generating novel phospholipid bilayers[151]. Enzymes that catalyse the generation of fatty acids, such as fatty acid synthase, or phospholipids, such as choline kinase, have important roles in lipid biosynthesis and can alter lipid compositions in cellular membranes in tumours as well as regulate cellular functions[23]. The development and function of different T cell subsets are closely linked to the predominant usage of fatty acid synthesis rather than fatty acid oxidation[179]. Short-chain fatty acids, such as butyrate and propionate, which are recognized mainly by G protein-coupled receptors such as GPR43 regulate the induction and homeostasis of T regulatory (T_{REG}) cells[168].

Cell activation increases rates of amino acid uptake by increasing expression of key amino acid transporters[180]. Branched-chain and aromatic amino acids, such as leucine, isoleucine, tryptophan and phenylalanine are used for *de novo* protein synthesis. Glutamine can be metabolized to lactate via glutaminolysis to support fatty acid synthesis[181].

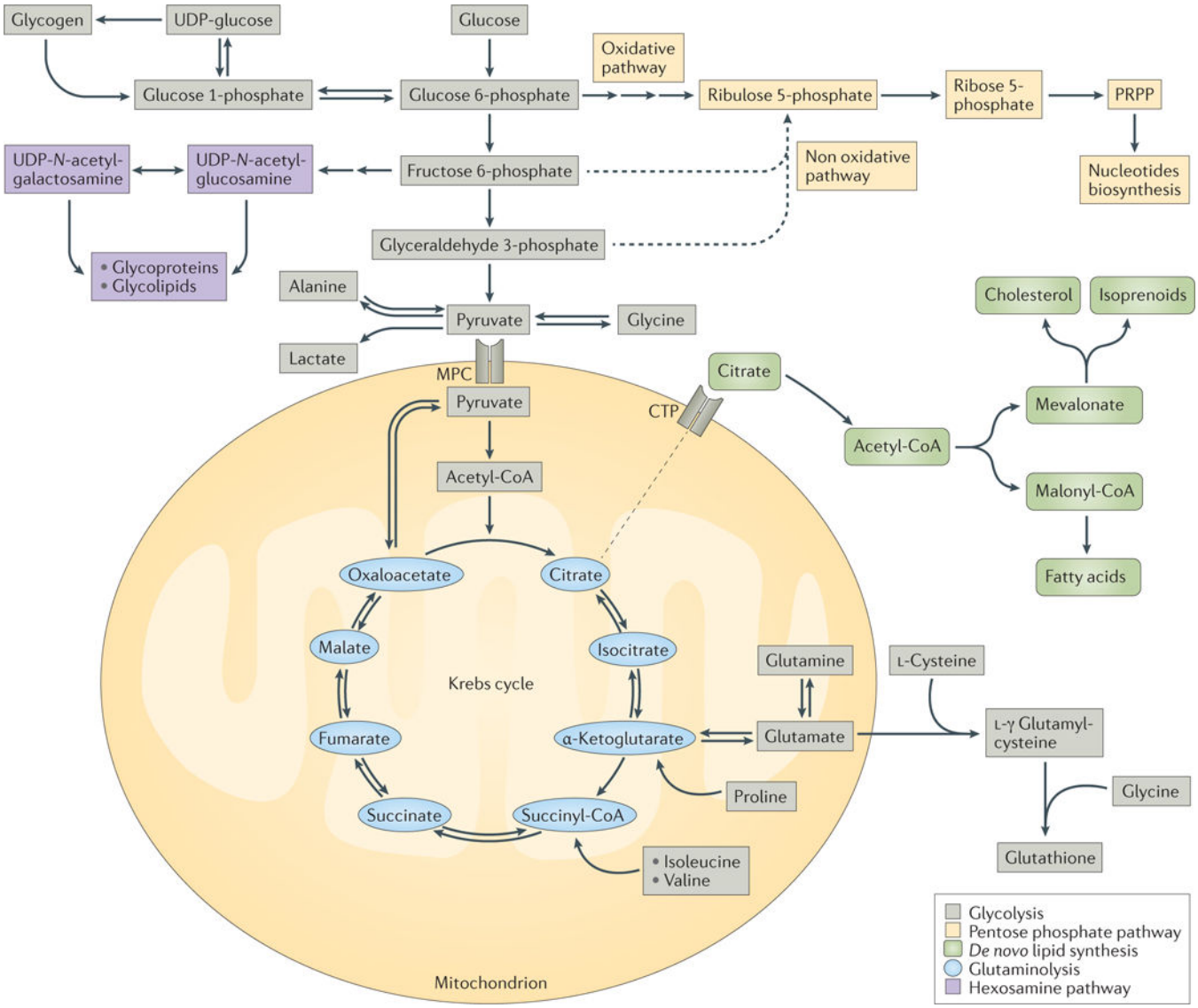

Figure 1. Overview of major metabolic pathways

Cellular metabolic pathways include synthesis of lipids, glycogen and nucleotides, glycolysis (the breakdown of carbohydrates and sugars to produce ATP and pyruvic acid) and the hexokinase pathway, which gives rise to substrates for the synthesis of glycoproteins and glycolipids. Mitochondrial oxidative phosphorylation (the Krebs cycle) is the sequence of reactions by which most living cells generate energy during the process of aerobic respiration. Glutaminolysis occurs partly in the mitochondrion and partly in the cytosol, and is an important energy source in tumour cells. CoA, coenzyme A; CTP, citrate transport protein; MPC, mitochondrial pyruvate carrier; PRPP, phosphoribosyl pyrophosphate; UDP, uridine diphosphate.

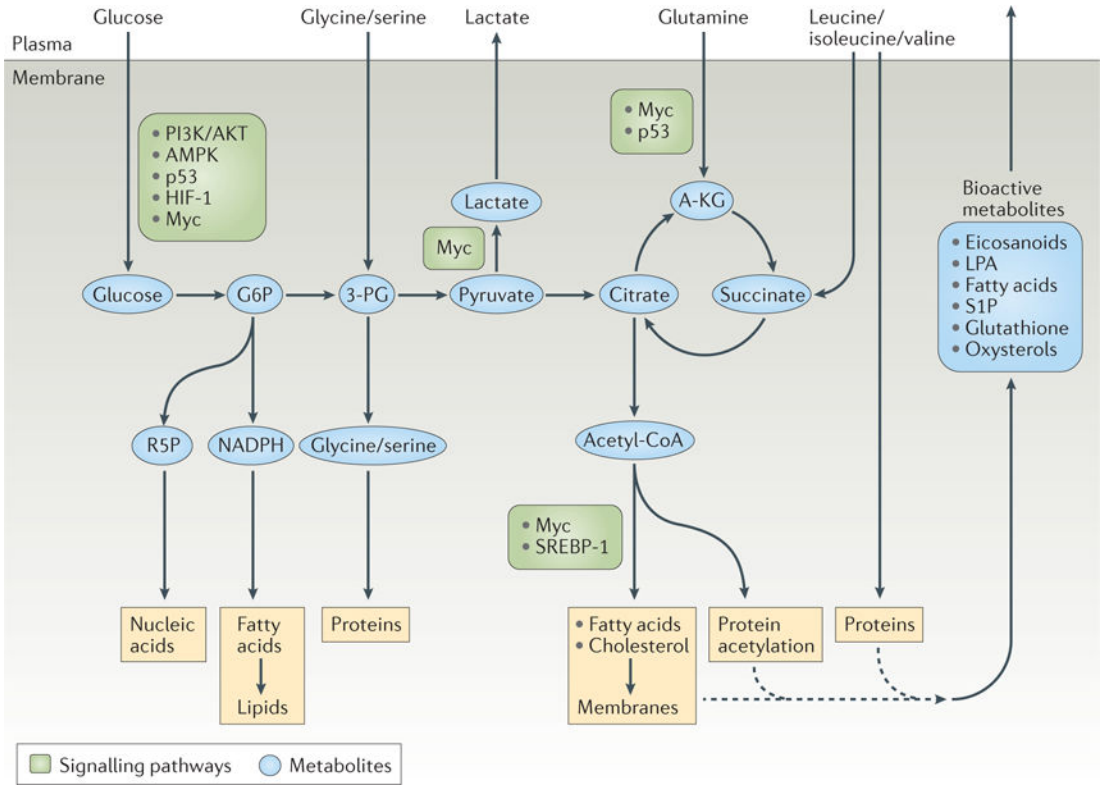

Figure 2. Metabolic alterations and signalling pathways involved in activated cells

Activated cells take up large amounts of glucose and glutamine and divert them to the pentose-phosphate pathway (PPP) and lipid biosynthesis, respectively. Coupled to an increased uptake of glycine, serine and branched chain amino acids (leucine, isoleucine and valine), which are required for protein synthesis, this diversion generates sufficient building blocks (nucleic acids, proteins and membranes) for proliferation. The increased generation of reactive oxygen species requires appropriate levels of antioxidants, most of which originate from the PPP. These metabolic changes generate bioactive metabolites that are secreted, and that also contribute to cell activation. Numerous signalling pathways are involved in metabolic changes in activated cells[182,183]. 3-PG, 3-phosphoglycerate; A-KG, α-ketoglutarate; AKT, protein kinase B; AMPK, AMP-activated protein kinase; CoA, coenzyme A; G6P, glucose-6-phosphate; HIF-1, hypoxia-inducible factor 1; L-AA, L-amino acid; LPA, lysophosphatidic acid; Myc, Myc proto-oncogene protein; p53, cellular tumour antigen p53; PI3K, phosphatidylinositol 4,5-bisphosphate 3-kinase; R5P, ribose-5-phosphate; S1P, sphingosine-1-phosphate; SREBP, sterol regulatory element-binding protein.

Table 1

Biological effects of key metabolites

Metabolite	Molecular effect	Examples of responsive cells	Cell response
Organic compounds			
Lactate	HIF-1α stabilization	• Tumour cells • DCs	• Angiogenic signalling • Differentiation to IL-10-producing DCs
Kynurenine	ND	T cells	T_H2 cell polarization
Succinate	HIF-1α stabilization	Macrophages	IL-1β expression
Acetyl coenzyme A	Histone-acetyltransferase activity	Tumour cells	Changes in energy homeostasis
Amino acids or peptides			
Branched amino acids	mTOR signalling	Macrophages	IL-1β expression
Glutathione	Antioxidant activity	T cells	T-cell proliferation
Lipids			
PIP3	Protein kinase B (AKT) signalling	Fibroblasts	Cell growth, proliferation and migration
Ceramides	ND	Tumour cells	Apoptosis
Sphingosine-1-phosphate	MAPK/PLC/PI3K signalling	T cells	Migration
LPA	G-protein-coupled receptor signalling	Fibroblasts, T cells	Proliferation, migration
Butyrate	Deacetylase inhibition	DCs and T cells	Induction of tolerogenic DCs, T-cell apoptosis
Oxysterols	Pro-inflammatory gene regulation	DCs	Enhanced DC immunogenicity
Nucleotides			
ATP	ND	DCs	Induction of tolerogenic DCs

DCs, dendritic cells; HIF-1α, hypoxia-inducible factor 1α; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; ND, not determined; PI3K, phosphatidylinositol 4,5-bisphosphate 3-kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PLC, phospholipase C; T_H2, type 2 T helper.