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Published in:
The Journal of Clinical Investigation

DOI:
[10.1172/JCI137552](https://doi.org/10.1172/JCI137552)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

de Heer, E. C., Jalving, M., & Harris, A. L. (2020). HIFs, angiogenesis, and metabolism: elusive enemies in breast cancer. *The Journal of Clinical Investigation*, 130(10), 5074-5087. <https://doi.org/10.1172/JCI137552>

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J Clin Invest. 2020;130(10):5074-5087. <https://doi.org/10.1172/JCI137552>.

Review Series

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HIFs, angiogenesis, and metabolism: elusive enemies in breast cancer

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Hypoxia-inducible factors (HIFs) and the HIF-dependent cancer hallmarks angiogenesis and metabolic rewiring are well-established drivers of breast cancer aggressiveness, therapy resistance, and poor prognosis. Targeting of HIF and its downstream targets in angiogenesis and metabolism has been unsuccessful so far in the breast cancer clinical setting, with major unresolved challenges residing in target selection, development of robust biomarkers for response prediction, and understanding and harnessing of escape mechanisms. This Review discusses the pathophysiological role of HIFs, angiogenesis, and metabolism in breast cancer and the challenges of targeting these features in patients with breast cancer. Rational therapeutic combinations, especially with immunotherapy and endocrine therapy, seem most promising in the clinical exploitation of the intricate interplay of HIFs, angiogenesis, and metabolism in breast cancer cells and the tumor microenvironment.

Introduction

Breast cancer is the cancer type with the highest prevalence and, despite therapeutic advances, still has the second highest cancer-related mortality rate in women (1). In breast cancer, low intratumoral O₂ levels (hypoxia) are associated with aggressive tumor behavior, metastasis, and resistance to therapy. The first in vivo measurements of oxygen content and subsequent observation of hypoxia in patients' breast tumors were described nearly 30 years ago (2). The transcription factor hypoxia-inducible factor 1 (HIF-1) was later characterized as the master regulator of cellular adaptation to hypoxia (3). The vital role of HIFs in every hallmark of cancer, in tumor progression, and in therapy resistance is now well established (4). Two fundamental processes that are especially dependent on HIFs are metabolic rewiring resulting in a more oxygen-independent nutrient metabolism, and angiogenesis, i.e., the growth of new blood vessels from preexisting vasculature. Targeting of key players in metabolic and angiogenic pathways in breast cancer has yielded disappointing results, the most notable being the lack of overall survival benefit of the antiangiogenic agent bevacizumab, which targets VEGF (5). This Review provides an overview of HIF-dependent reprogramming of angiogenic and metabolic pathways in breast cancer and discusses novel approaches and challenges in the clinical translation of this knowledge into successful treatment strategies.

HIF activity in breast cancer

Active HIF is composed of the constitutively expressed HIF-1 β subunit, an O₂-dependent HIF α isoform, and essential cofactors. HIF induces transcription of target genes by binding to hypoxia-responsive elements (HREs) in promoters. As in all mammalian cells, in breast cancer, HIF α stability and corresponding HIF activity are greatly increased in hypoxia (Figure 1). In normoxia, HIF activity is repressed through proteasomal degradation of HIF α by the O₂-dependent prolyl hydroxylase domain (PHD) proteins and the von Hippel-Lindau (VHL) protein, and/or by inhibition of HIF α binding to essential cofactors by factor inhibiting HIF-1 (FIH-1) (6). Downstream targets of the HIF α isoforms (HIF-1 α and HIF-2 α) only partially overlap, and in breast cancer, HIF-1 α is the predominantly (over)expressed isoform (7, 8). Recently, specific roles for HIF-2 α in breast cancer progression, mediated upstream by the transcription factor FOXA1, and in angiogenesis have been identified (9, 10). In human breast tumors, HIF-1 α is already overexpressed in precursor lesions (ductal carcinoma in situ [DCIS]) and early-stage breast cancer, and these levels strongly correlate with tumor grade and invasion (11). HIF-1 α foci are predominantly observed surrounding necrotic areas such as the generally hypoxic tumor core.

Common genetic alterations in breast cancer, such as loss of the tumor suppressors PTEN, p53, or BRCA1 and hyperactivation of the PI3K/Akt/mTOR or MAPK pathway, increase HIF α transcription, translation, or stability independently of O₂ levels (refs. 4, 12, 13, and Figure 1). Human epidermal growth factor receptor 2 (HER2; overexpressed in 15%–30% of human breast cancers) and estrogen receptor- α (ER α ; positive in approximately 70% of breast cancers) increase HIF α levels through increased PI3K/Akt/mTOR signaling (14, 15). ER α also directly induces HIF-1 α , but not HIF-2 α , expression through an estrogen response element in the *HIF1A* promoter (16, 17).

Conflict of interest: ALH serves in a paid advisory role for Curve Therapeutics. MJ serves in an advisory role for Bristol Myers Squibb, Merck & Co., Novartis, AstraZeneca, Tesaro, and Pierre Fabre (fees paid to the institution).

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Reference information: *J Clin Invest.* 2020;130(10):5074–5087.

<https://doi.org/10.1172/JCI137552>.

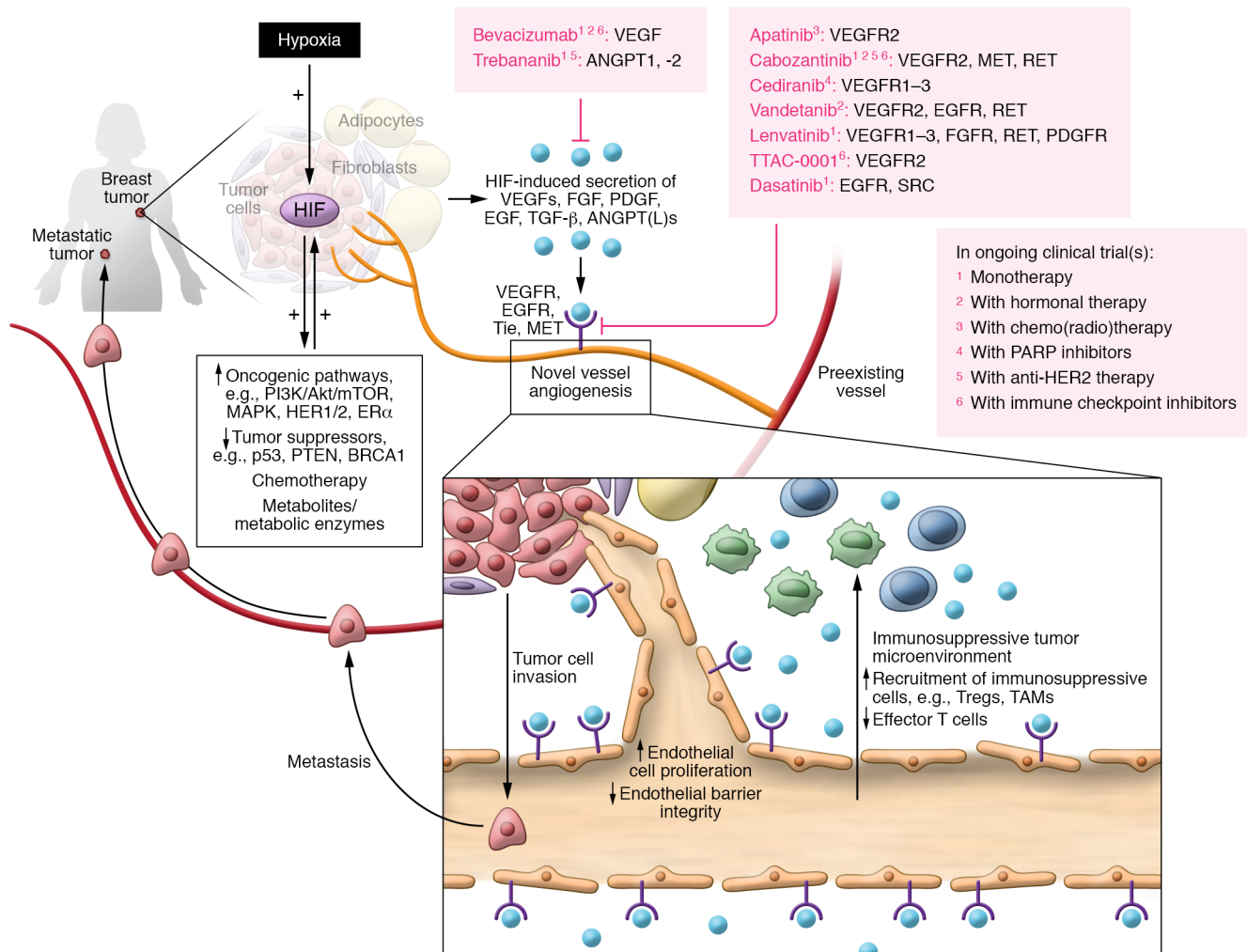


Figure 1. Schematic overview of HIFs and HIF-induced angiogenesis in breast cancer. HIF is stimulated by both hypoxia and O₂-independent oncogenic, metabolic, and therapeutic factors. HIF drives angiogenesis by inducing secretion of proangiogenic growth factors by tumor cells and stromal cells, such as adipocytes and fibroblasts. The newly formed vasculature is disorganized and leaky, which facilitates tumor cell invasion and metastasis, impairs drug delivery, and further aggravates hypoxia in the tumor and the microenvironment. Angiogenic growth factors also contribute to an immunosuppressive tumor microenvironment, particularly by increasing recruitment of immunosuppressive cells. Compounds targeting angiogenic key players are listed in pink text. The key indicates their furthest stage of development in the breast cancer setting and evaluation in clinical trial(s) as monotherapy or as combination therapy. ANGPT(L), angiotensin-like protein; BRCA, breast cancer gene; ER, estrogen receptor; FGFR, fibroblast growth factor receptor; HER, human epidermal growth factor receptor; MET, hepatocyte growth factor receptor; PARP, poly (ADP-ribose) polymerase; PTEN, phosphatase and tensin homolog; RET, rearranged during transfection; TAM, tumor-associated macrophage.

HIF-1 α immunohistochemistry in patient breast tumors correlates with ER α expression and HER2 positivity in some, but not all, studies (11, 18–22). High HIF-1 α levels are consistently reported in triple-negative breast cancer (TNBC), the poor-prognosis subtype that lacks (over)expression of hormonal and HER2 receptors (23–25). TNBC patients show especially high uptake of the PET tracer ¹⁸F-fluoromisonidazole, which selectively accumulates in hypoxic cells (26), and TNBC cells carry a hypoxia gene signature in normoxic conditions (27). In TNBC, there is a high prevalence of p53 loss, *PTEN* mutations, and EGFR overexpression, all of which can lead to increased HIF activity (25). The transcription factor X-box binding protein 1 may regulate HIF responses in TNBC (28, 29). The lack of elevated *HIF1A* mRNA levels in TNBC cells implies that important post-transcriptional mechanisms also contribute to the high HIF

activity (27). Interestingly in this respect, intracellular depletion of the amino acid cysteine stabilizes HIF-1 α in TNBCs in normoxia and was associated with dysfunctional PHDs and paracrine glutamate signaling (23).

Multiple other metabolites and HIF-induced metabolic enzymes are involved in feed-forward loops with HIF activity in normoxia, including ROS, acetyl-CoA synthetase 2 (ACSS2), and mitochondrial proteins such as CHCHD4 (refs. 4, 30–33, and Figure 1). HIF α expression, stability, and effector function at HREs are additionally influenced by other (bidirectional) processes such as epigenetics, the circadian rhythm, noncoding RNAs, and HIF-dependent secretion of microvesicles by tumor cells or cells in the tumor microenvironment (TME) (9, 34–38). For instance, tumor-associated macrophages secrete vesicles containing the long noncoding RNA H1SLA, which blocks the PHD/HIF-1 α interaction and induces glycolysis in

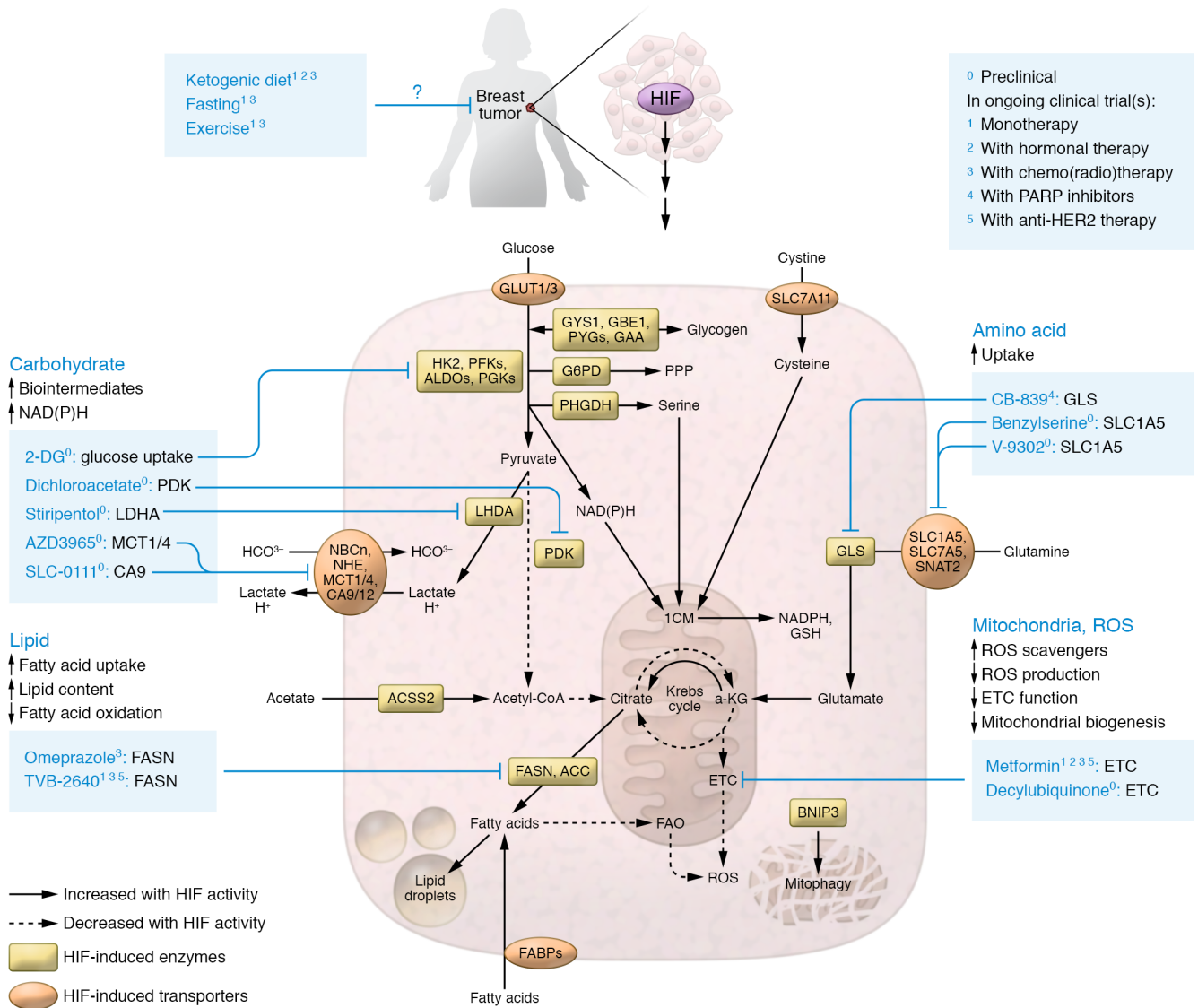


Figure 2. HIFs drive reprogramming of multiple metabolic pathways in breast cancer. In general, HIF activity increases glycolysis and related carbohydrate pathways (e.g. pentose phosphate pathway and glycogen metabolism) as well as lactate export while suppressing mitochondrial O₂-dependent metabolism. Amino acid, acetate, and fatty acid uptake are increased to fuel processes that are essential for formation of ROS scavengers and Krebs cycle intermediates. This metabolic rewiring not only allows rapid proliferation and protects cells from ROS-induced damage but also contributes to formation of breast cancer stem cells and generation of an acidic and nutrient-depleted immunosuppressive microenvironment. Drugs with their respective targets or nonpharmacological, patient-centered strategies that target the rewired metabolism in breast cancer are listed in blue text. The key notes their furthest stage of (pre)clinical development in the breast cancer setting and/or evaluation in clinical trial(s) as monotherapy or as combination therapy. 1CM, one-carbon metabolism; 2-DG, 2-deoxyglucose; ACC, acetyl-CoA carboxylase; ACSS, acetyl-CoA synthetase; ALDO, aldolase; BNIP3, BCL2- and adenovirus E1B 19-kDa-interacting protein 3; CA, carbonic anhydrase; ETC, electron transport chain; FABP, fatty acid-binding protein; FAO, fatty acid oxidation; FASN, fatty acid synthase; G6PD, glucose-6-phosphate dehydrogenase; GAA, α-1,4-glucosidase; GBE, glycogen branching enzyme; GLUT, glucose transporter; GSH, glutathione; GYS, glycogen synthase; HK, hexokinase; α-KG, α-ketoglutarate; LDHA, lactate dehydrogenase A; MCT, monocarboxylate transporter; NBC, Na⁺-bicarbonate cotransporter; NHE, Na⁺/H⁺ exchanger; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PHGDH, phosphoglycerate dehydrogenase; PPP, pentose phosphate pathway; PYG, glycogen phosphorylase; SLC, solute carrier; SNAT, sodium-coupled neutral amino acid transporter.

normoxic breast cancer cells (35). HISLA secretion itself is increased by high extracellular lactate, demonstrating the intricate bidirectional pathways regulating HIFα expression (29, 36, 38).

HIF-induced angiogenesis in breast cancer

O₂ diffusion from the nearest blood vessel, limited to a distance of 100 to 150 μm, typically supports tumor growth until it reach-

es a volume of 1–2 mm³. Angiogenesis allows tumors to continue growing beyond sizes at which diffusion-mediated O₂ and nutrient supplies fall short. HIF activity is the major driver of angiogenesis. The sprouting microvasculature in the TME is disorganized and leaky, in contrast to angiogenesis in normal tissue, and amplifies intratumoral hypoxia and favors metastatic spread while diminishing drug delivery and hampering antitumor immune responses

Table 1. Selected studies reporting prognostic and/or predictive value of HIF and HIF targets in metabolism and angiogenesis in breast cancer patients

Biomarker	Method	Prognostic for	Predictive for
General HIF			
HIF-1 α	IHC	OS (18, 20) DFS (18, 20)	Neoadjuvant chemotherapy (22, 103, 105) Anti-estrogen (175)
HIF-2 α	IHC	DSS (176) RFS (176) OS (177)	-
miR-210	RNA sequencing	OS (29) Time to metastasis (29)	-
Hypoxia gene signature	RNA sequencing Microarray	OS (27, 99, 100)	Antiangiogenic (80)
(Peri)tumoral oxygen saturation	Diffuse optical spectroscopy imaging ¹⁸ F-MISO PET/CT	-	Neoadjuvant chemotherapy (178 ^A , 179) Anti-estrogen (180)
Metabolism			
CA9	Serum measurement	PFS (181, 182)	(Neo)adjuvant chemotherapy (183, 184)
pH regulation	IHC	DFS (182, 183) OS (182) DSS (183)	
Glycolysis	IHC (GLUT1, HK2 etc.)	DFS (96, 185)	(Neo)adjuvant anti-HER2 + chemotherapy (115, 116, 186)
Carbohydrate metabolism	¹⁸ F-FDG PET/CT imaging	OS (96)	Neoadjuvant chemotherapy (103, 187, 188)
NDRG1	RNA sequencing	RFS (81, 112)	Antiangiogenic (80)
Fatty acid metabolism	IHC	OS (112)	
SLC7A5	RNA sequencing	RFS (111, 112)	-
Amino acid metabolism	IHC	OS (111, 112) DSS (113)	
SLC1A5	IHC	DFS (72)	-
Amino acid metabolism	RPPA		
SNAT2	Gene array	-	Anti-estrogen (66)
Amino acid metabolism			
PHGDH	RNA sequencing	RFS (75)	-
Amino acid/ROS metabolism			
Angiogenesis			
CXCR4	IHC/IS/WB	PFS (189) ^A OS (189) ^A	-
Microvessel density	IHC	RFS (98 ^A , 190) OS (98 ^A , 190)	-
VEGFA	IHC	DFS (191)	-
VEGFC	IHC	OS (191, 192) ^A DFS (191, 192) ^A	-
VEGFR1	IHC	DFS (191)	-
MET	IHC/IS/RPPA/WB/FISH	PFS (189) ^A OS (124) ^A RFS (124) ^A	Adjuvant chemoradiotherapy (184)

^AMeta-analysis. CA, carbonic anhydrase; DFS, disease-free survival; DSS, disease-specific survival; ¹⁸F-FDG, ¹⁸F-fluorodeoxyglucose; FISH, fluorescence in situ hybridization; ¹⁸F-MISO, ¹⁸F-fluoromisonidazole; GLUT, glucose transporter; HK, hexokinase; IS, immunostaining; MET, hepatocyte growth factor receptor; NDRG, N-myc downstream regulated gene; OS, overall survival; PFS, progression-free survival; PHGDH, phosphoglycerate dehydrogenase; RFS, relapse-free survival; RPPA, reverse-phase protein array; SLC, solute carrier; SNAT, sodium-coupled neutral amino acid transporter; WB, Western blot.

(Figure 1 and refs. 39, 40). Breast cancer angiogenesis requires a well-balanced interplay between classical HIF-regulated angiogenic inducers (e.g., VEGF), angiogenic receptors (e.g., VEGFR, angiopoietin [ANGPT] receptors), and components of cell adhesion and extracellular matrix remodeling (41–43). Novel mediators of tumor angiogenesis are rapidly being identified (36). The long noncoding mRNA RAB11B-AS1 was increased in hypoxia in a HIF-2 α -depen-

dent manner and increased breast cancer angiogenesis and metastatic potential by recruiting RNA polymerase to VEGFA and angiopoietin-like 4 (ANGPTL4) (10). ANGPTL4 itself is a HIF-1 target that promotes lung metastasis when overexpressed in breast cancer cells (44). A recent breast cancer study in mice pointed toward adipocytes as an additional important source of ANGPTL4, and its secretion was synergistically controlled by hypoxia and IL-1 β

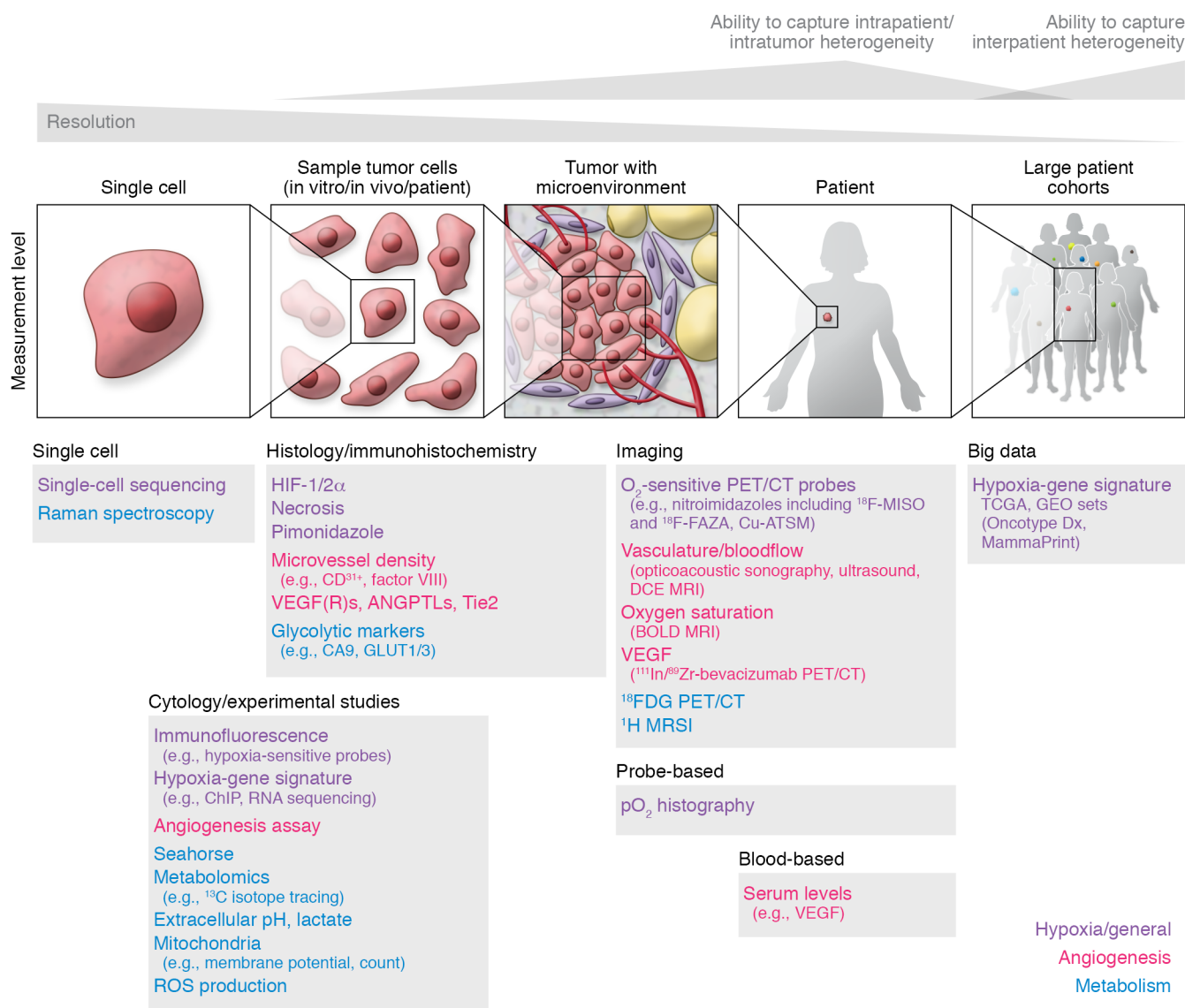


Figure 3. Approaches to measure HIF activity, cancer angiogenesis, and metabolism. Depending on the method and the scale of application, various degrees of detail, intratumor and intrapatient heterogeneity, and interpatient heterogeneity can be captured. ANGPTL, angiopoietin-like protein; BOLD, blood oxygenation level-dependent; CA, carbonic anhydrase; Cu-ATSM, copper(II)-diacetyl-bis(*N*⁴-methylthiosemicarbazone); DCE, dynamic contrast-enhanced; ¹⁸F-FAZA, ¹⁸F-fluoroazomycin arabinoside; ¹⁸F-FDG, ¹⁸F-fluorodeoxyglucose; ¹⁸F-MISO, ¹⁸F-fluoromisonidazole; GEO, Gene Expression Omnibus; MRSI, magnetic resonance spectroscopic imaging; PET/CT, positron emission tomography/computed tomography; TCGA, The Cancer Genome Atlas; Tie2, TEK receptor tyrosine kinase 2.

(45, 46). Similarly, other studies reveal HIF-mediated release of (exosomal) proinflammatory and proangiogenic substances such as TGF- β and prostaglandin E₂ by breast cancer cells, adipocytes, infiltrating CD8⁺ T cells, and other stromal cells (36, 39, 47–49), suggesting an intricate interplay between HIFs, proinflammatory factors derived from tumor and various TME cells, and angiogenesis that has yet to be fully elucidated.

HIF-induced metabolic reprogramming in breast cancer

Carbohydrate metabolism. HIF-1 activity induces a shift from respiratory, O₂-dependent mitochondrial metabolism toward glycolytic, O₂-independent metabolism through upregulation of nearly all glycolytic

enzymes and redirection of pyruvate from entry into the Krebs cycle toward lactate production (refs. 4, 6, and Figure 2). Pyruvate dehydrogenase kinase (PDK) is a HIF-induced key regulator of lactate production via inhibition of pyruvate dehydrogenase (PDH), which rapidly inhibits the first step of the Krebs cycle during hypoxia (50).

These effects of HIF, which occur in *hypoxia*, are often confused with the Warburg effect, which is defined as *aerobic* glycolysis and is essential for formation of sufficient intermediates and reducing equivalents for rapid cell division and survival. Although normoxic HIF can mimic these effects, and HIF α may be upregulated by oncogenes, multiple other mechanisms are relevant, e.g., MYC and RAS (51). HIF not only induces glucose transporter (GLUT) expression for uptake of extracellular glucose

Table 2. Specific rationales for exploring synergy between approved breast cancer therapies and (novel) therapies targeting HIF/hypoxia, angiogenesis, and HIF-related metabolic reprogramming, as proposed or tested in the preclinical setting

Approved therapy	Mechanism of action	Main rationale(s) for combination therapy	Refs
Immune checkpoint inhibition	Prevents inactivation of TILs by blocking immune checkpoints (PD-L1, PD-1, CTLA-4)	Exploit PD-L1 upregulation that is induced by HIF ^a Enhance immune cell infiltration (TILs, dendritic cells) by normalizing vasculature ^b Decrease (VEGF-mediated) induction of immunosuppressive subsets (e.g., Tregs, M2 macrophages) ^b Exploit PD-1 and CTLA-4 upregulation that is induced by anti-VEGF treatment ^b Decrease immunosuppressive TME by normalizing extracellular pH and suppressing tumor nutrient uptake ^c	119, 147, 149, 150, 193–197
Radiotherapy	Induces lethal DNA damage by ROS	Enhance tumor oxygenation and ROS production by normalizing vasculature ^b	198, 199
Chemotherapy	Induces lethal DNA damage	Overcome/prevent (multidrug) resistance and BCSC induction ^a Increase chemotherapy delivery(?) ^b Concurrent hits in multiple cancer hallmarks ^b	39, 83, 106
Antiestrogen therapy	Blocks constitutive growth signals from overexpressed ER (ER antagonists) or endogenous estrogen production (aromatase inhibitors)	Overcome/prevent endocrine resistance by blocking compensatory HIF upregulation ^a Decrease endocrine resistance by blocking amino acid metabolism ^c	9, 66, 67, 113, 114, 155
HER2-targeted therapy	Blocks constitutive growth signals from overexpressed HER2 and/or directs chemotherapy delivery	Overcome/prevent T-DM1 resistance by reversing hypoxia-induced caveolin-1 relocation and drug internalization ^a	200

^aRationale for combination with therapies targeting HIF/hypoxia. ^bRationale for combination with therapies targeting angiogenesis. ^cRationale for combination with therapies targeting HIF-related metabolic reprogramming. BCSC, breast cancer stem cell; CTLA-4, cytotoxic T lymphocyte-associated protein 4; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; T-DM1, trastuzumab-emtansine; TIL, tumor-infiltrating lymphocyte; TME, tumor microenvironment.

but also increases glycogen synthesis and breakdown as an additional glucose source to sustain glycolytic and pentose phosphate flux. Breast cancer glycogen metabolism has been implicated in improved ROS scavenging, survival after reoxygenation, cell migration, and radioresistance (52).

HIF-induced membrane expression of lactate, H⁺, and HCO₃⁻ transporters is crucial for survival of hypoxic tumor cells by preventing intracellular pH reduction caused by lactate production, thereby allowing continuously high glycolytic rates and contributing to an acidic, immunosuppressive TME (53–55). While normal breast tissue does not express carbonic anhydrase 9 (CA9), it is widely overexpressed from DCIS (56) to invasive ductal carcinoma (57, 58) and lymph node metastases (59, 60). CA9 expression correlates well with tumor HIF-1 α activity and is particularly pronounced in perinecrotic tumor regions, high-grade breast cancers, and TNBC (54, 58, 61). Besides the canonical CA function of catalyzing the interconversion of CO₂ and water to HCO₃⁻ and H⁺ (53, 54), the noncatalytic domain of CA9 interacts with monocarboxylate transporters (MCTs) 1 and 4 in human breast cancer tissue, facilitating MCT-mediated lactate and H⁺ efflux in preclinical models (62–65).

Amino acid metabolism. Amino acids, acetyl-CoA, and Krebs cycle intermediates are indispensable for nucleoside, lipid, and glutathione formation. To compensate for the reduced influx of pyruvate into the Krebs cycle, hypoxic cancer cells rely on uptake of amino acids such as glutamine and cystine to fuel this cycle. Glutamine, especially, has a central role in cancer cell metabolism. The amino acid importers SNAT2 (which imports neutral α -amino acids including glutamine and alanine), solute-linked carrier family A1 member 5 (SLC1A5, also known as alanine, serine, cysteine transporter 2 [ASCT2], importing neutral amino acids, especially glutamine), SLC7A11 (a cystine-glutamate

antiporter), and SLC7A5 (which mediates import of large neutral amino acids including leucine and tyrosine) and the enzyme glutaminase (GLS), which catalyzes glutamine-to-glutamate conversion, are all upregulated by HIF (refs. 66–70 and Figure 2). SLC1A5 was recently shown to be a HIF-2 target (68) and is especially overexpressed in TNBC. In vitro and in vivo SLC1A5 knockdown inhibits growth in TNBC, but not ER α ⁺ breast cancer, sensitizes TNBC cells to chemotherapy, and is lethal in TNBCs that do not show a flexible compensatory increase in other amino acid transporters (71–73).

Serine, a nonessential amino acid derived from the glycolytic intermediate 3-phosphoglycerate, and cysteine are key for NADPH and glutathione formation in hypoxic breast cancer cells (70, 74, 75). Phosphoglycerate dehydrogenase (PHGDH) and all other downstream enzymes in serine, cysteine, and downstream mitochondrial one-carbon metabolism are upregulated by HIF (70, 75). PHGDH knockdown in breast cancer cell lines reduces NADPH and glutathione levels, increases ROS levels, impairs metastatic potential by reducing breast cancer stem cells (BCSCs), and increases chemotherapy sensitivity. In contrast, breast cancer cell proliferation and growth are only impaired upon PHGDH knockdown in low-serine culture medium or in cell lines with a PHGDH copy number gain (a small subset of TNBC). This implicates that breast cancer cells depend heavily on serine metabolism for ROS scavenging but are only dependent on it for biomass in case of intrinsic baseline PHGDH overexpression or serine-limiting environmental conditions (75, 76).

Lipid metabolism. Elevated levels of lipids and upregulation of fatty acid (FA) synthase (FASN) in breast cancer were the first observations consistent with the now well-established importance of lipid metabolism in cancer cells (77, 78). Cancer cells require FAs and lipids as building blocks for cell membranes, signaling

molecules, energy, and reducing capacity during reoxygenation (77). HIF-1 activity represses FA oxidation, thereby reducing ROS generation, and upregulates FASN, lipin 1, acetyl-CoA carboxylase (ACC), and others for lipid and FA synthesis (Figure 2). Nevertheless, hypoxic cells are thought to preferably derive FAs from increased uptake by upregulating FA-binding proteins (FABPs), needed for FA uptake and intracellular trafficking, and predominantly use de novo lipid and FA synthesis from acetyl-CoA in nutrient-deprived conditions (77). Acetyl-CoA can be supplied through import of acetate, which is directly converted to acetyl-CoA in the cytoplasm by the HIF target ACS2 (6, 71, 77, 79).

The HIF-regulated N-myc downstream regulated gene 1 (NDRG1) is predominantly overexpressed in perinecrotic areas and ER α -breast cancer and is predictive for bevacizumab response and prognostic for survival (80, 81). Homozygous loss of function of NDRG1 in humans causes a neurological disorder with nerve demyelination, and manipulation of NDRG1 in breast cancer cell lines deregulated lipid droplet storage, although its exact metabolic function and discrepancies in its reported effects on migration and breast cancer progression require further clarification (81, 82).

Mitochondrial and ROS metabolism. ROS are produced due to dysfunction of the mitochondrial electron transport chain under hypoxic or hyperoxic conditions. In fact, in experimental hypoxia and HIF-KO models the prime cause of tumor cell death is ROS, rather than absolute O₂ deficiency (83). HIFs keep intracellular ROS levels in check by increasing BCL2- and adenovirus E1B 19-kDa-interacting protein 3/Nip3-like protein X/FUN14 domain containing 1-mediated (BNIP3/NIX/FUNDC1-mediated) mitophagy, suppressing mitochondrial biogenesis, redirecting metabolic pathways to mitochondria-independent alternatives, and increasing production of the ROS scavenger glutathione and the reducing equivalent NAD(P)H (refs. 83–85 and Figure 2).

HIF-mediated suppression of nuclear respiratory factor 1 (NRF-1) decreases transcription of mitochondrial genes, and inhibition of the NRF-1 degrader SIAH2 (seven in absentia homolog 2; an E3 ubiquitin ligase) is associated with elevated NAD⁺/NADH ratios, succinate dehydrogenase activity, and increased mitochondrial mass (85, 86). Besides favoring breast cancer viability and growth, sublethal ROS levels stimulate HIF activity and induce cellular transformation into a BCSC phenotype, characterized by ongoing self-renewal capacity, stem cell markers such as aldehyde dehydrogenase (ALDH), and involvement in relapse and therapy resistance (83, 87). Moreover, HIF-1-dependent BCSC enrichment is observed upon chemotherapy treatment, and the majority of murine metastatic breast cancer cells exhibit a post-hypoxic, ROS-resistant phenotype even after reoxygenation (87–90).

Biomarkers of HIF-regulated metabolism and angiogenesis

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (91). Biomarkers can be prognostic, i.e., providing information on survival outcomes irrespective of the received treatment, and/or predictive, i.e., providing information on likelihood of treatment response. For instance, presence or absence of lymph node metastases is a strong prognostic but

not a predictive marker, whereas the established breast cancer biomarkers HER2 overexpression and ER α expression are validated as prognostic as well as predictive biomarkers for response to HER2-targeted and hormonal therapy, respectively.

Multiple HIF-regulated angiogenic and metabolic tissue markers — either alone or in combination — have been implicated as prognostic for overall and progression-free survival and/or predictive for breast cancer chemotherapy, hormonal therapy, and kinase-targeted therapies (Table 1). Nevertheless, repeatability and clinical implementation of immunohistochemical markers are notoriously challenging, and study outcomes have been highly variable. Moreover, biopsy-based biomarkers are limited by sampling bias because they represent only a single part of a single tumor lesion. Imaging techniques can overcome this limitation by providing both static and dynamic whole-body measurements, albeit limited by their resolution. Noninvasive imaging approaches that measure real-time tumor blood flow or hemoglobin oxygen saturation or visualize trapped hypoxia-sensitive radioactive probes using PET could replace microvessel density (MVD) assessment, and whole-body ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) PET/CT imaging may replace GLUT1 immunohistochemistry (refs. 92, 93, and Figure 3). The sections below discuss the most recent developments and previous studies that have been pioneering and/or included relatively large populations.

Prognostic markers. Tumor hypoxia has been measured mainly by determination of HIF-1 α expression and surrogates such as MVD and CA9 that are more stable than HIF-1 α itself, which has a half-life of ≤ 5 minutes upon reoxygenation (3, 94). Presence of a hypoxic phenotype is prognostic for relapse and poor survival across breast cancer subtypes and stages, corroborated by well-powered pan-cancer meta-analyses (95, 96). The relative risks of high expression of HIF-1 α , MVD, VEGF, CA9, and other hypoxic markers are only moderate compared with known clinical prognostic factors that already represent the aggressive phenotype associated with HIFs (e.g., receptor status, lymph node status, tumor grade). Contradictory results among studies are likely due to inconsistent multivariate correction, methodological differences in antibodies and targets for visualizing vascular endothelium (e.g., CD31⁺, PDGF, factor VIII), variable scoring methodologies (e.g., manual vs. automated, nuclear vs. diffuse HIF α staining), and different stratification cutoffs (97, 98).

Rather than pinpointing of one marker, breast cancer HIF activity is increasingly captured by large-scale RNA sequencing in prognostic hypoxia-signature gene panels that contain components across multiple pathways downstream of HIF (27, 99, 100). This approach enhances the power to detect biologically relevant processes and guides discovery of new therapeutic targets and markers. Derived signatures can be validated in data sets publicly available online and in future studies. Genome-wide analysis of germline variations in almost 100,000 breast cancer patients in different cohorts revealed no major novel individual prognostic factors, whereas a network analysis identified the module “cell growth and angiogenesis” as prognostic for ER α - but not ER α ⁺ breast cancer (101). One of the four components in this module was *CHCHD4*, which encodes a mitochondrial protein involved in HIF-1 α stability and regulation of mitochondrial respiratory chain in tumor cell adaptation to hypoxia (33, 102).

Predictive markers. It is generally acknowledged that tumor hypoxia and multiple HIF-related markers predict worse response to chemoradiotherapy, and neoadjuvant studies have shown lower pathological complete response (pCR) rates in patients with high baseline HIF α expression (22, 103–105). Several biological mechanisms explaining the negative correlation of HIF activity with chemoradiotherapy response have been described. Cytotoxicity of radiotherapy depends on ROS-induced catastrophic DNA damage, which therefore requires at least some O₂. Additionally, the dysfunctional blood supply in hypoxic tumor regions may reduce delivery of cytotoxic drugs, and moreover, HIF upregulates P-glycoprotein, also called multidrug resistance protein 1 (39, 42, 106). Finally, HIFs and chemotherapy both induce chemotherapy-resistant BCSCs (83, 87, 107). The gene panels Oncotype DX and MammaPrint are prognostic for survival and predictive for benefit from adjuvant chemotherapy in ER α ⁺HER2⁻ breast cancer patients and are used in clinical decision making. Both panels consist of gene sets that include known HIF targets and/or players in tumor metabolism and angiogenesis such as matrix metalloproteinase 9 (*MMP9*) and egl-9 family hypoxia-inducible factor 1 (*EGLN1*), encoding PHD2 (23, 108). However, two of the control genes, *GAPDH* and *TFRC* (transferrin receptor), are well-validated HIF-1 targets, implying that differences driven by hypoxic tumor biology may be missed in these analyses (109, 110).

High expression of HIF-1 α and the HIF-regulated amino acid importers SNAT2, SLC1A5, and SLC7A5 has been associated with shorter survival in the ER α ⁺ highly proliferative subtype (luminal B) and resistance to the antiestrogen therapies tamoxifen and aromatase inhibitors (66, 111–114). SNAT2 overexpression in hypoxic breast cancers is HIF-1 α - and HIF-2 α -dependent and strongly corresponds with *HIF1A* mRNA expression and wider hypoxia gene signatures. *SNAT2* has overlapping binding sites for HIF-1 α and ER α , and during tamoxifen treatment, which abolished ER α signaling, HIF-1 α could replace this signaling and increase SNAT2 expression under hypoxic conditions. *SNAT2* knockdown reversed tamoxifen resistance and dampened signaling through the mTOR pathway, the latter being a known resistance mechanism to antiestrogen therapy (66). Other reports also describe a HIF-2 α and/or SLC7A5/mTOR regulatory axis underlying endocrine resistance (9, 67). In addition, contralateral breast cancers developing during adjuvant tamoxifen treatment, i.e., indicating intrinsic antiestrogen resistance, were more often HIF-1 α -expressing than treatment-naïve contralateral tumors (21).

The backbone of systemic therapy in breast cancer patients overexpressing HER2 are drugs that suppress the downstream oncogenic PI3K/Akt/mTOR and MAPK signaling pathways through HER2 inhibition and, in the case of the antibody-drug conjugate trastuzumab-emtansine (T-DM1), additionally deliver localized chemotherapy. The intensity of HER2 expression as determined by immunohistochemistry or FISH in tumor biopsies is the strongest predictive factor for therapy response, but intrinsic or induced resistance is a major clinical challenge that is not predicted by expression alone. ¹⁸F-FDG uptake on PET/CT is prognostic in the neoadjuvant and the metastatic setting for, respectively, pCR and early treatment failure (after approximately 2 cycles) (115, 116). Other markers of HIF-1/2 α expression or downstream

metabolic or angiogenic targets have not been reported as predictive for response or resistance to HER2-targeted therapy.

The initial progression-free survival (PFS) gain in breast cancer demonstrated for the VEGF-targeting antibody bevacizumab did not translate into an overall survival (OS) benefit. It was subsequently reasoned that only patients with especially deregulated and widespread tumor microvasculature might benefit from bevacizumab-induced vessel normalization. However, in retrospective analyses, intuitively logical biomarkers correlated with pCR rates and normalization of tumor vasculature in some cases but did not predict final clinical outcomes. Evaluated biomarkers include high baseline MVD, high volume transfer constant on dynamic contrast-enhanced MRI, elevated expression of proangiogenic factors (e.g., VEGF, VEGFR, and Tie2 measured immunohistochemically or in patients' serum), and, more recently, NDRG1 and panels representing DNA methylation status or hypoxia gene sets in HER2⁻ breast cancer patients on neoadjuvant bevacizumab plus chemotherapy (5, 80, 117–119). Multiple alternative vascular markers are being evaluated in different cancer types, e.g., the vascular co-option players stromal-derived factor 1 α and CXCR4, and ANGPT2 (5, 39).

Targeting hypoxia, angiogenesis, and metabolism in breast cancer

In breast cancer, hypoxia mediates aggressive, metastatic, and therapy-resistant disease, making it an attractive target for novel (combination) therapies (Table 2). Hypoxic tumor cells can be targeted directly, for example by use of hypoxia-activated prodrugs or by specific targeting of HIFs (reviewed in ref. 120). Strategies to target HIFs include downregulating HIF α protein expression, blocking HIF α -HIF β dimerization or essential cofactor binding, and preventing binding of HIF to HREs. It has, however, been challenging to develop specific, potent HIF-1 α inhibitors with suitable pharmacological properties for clinical evaluation. Review of ClinicalTrials.gov does not show any currently active breast cancer trials testing drugs directly targeting HIFs, although there are ongoing studies on (novel) inhibitors of mTOR (e.g., TAK-228), PI3K (e.g., BKM-120 or BYL-719), and histone deacetylases (vorinostat), which all indirectly target HIF signaling. Instead, therapeutic strategies often focus on consequences of hypoxia, including angiogenesis and reprogrammed metabolism, as discussed below (see also Figure 1 and Figure 2).

Therapeutic strategies targeting angiogenesis. The largest body of evidence is available for bevacizumab, a monoclonal antibody that blocks VEGF. As mentioned, in metastatic breast cancer only modest benefits in PFS were achieved, not translating into OS benefit, resulting in FDA withdrawal after initial approval. Targeting VEGF signal transduction with tyrosine kinase inhibitors is another strategy, but results in metastatic breast cancer are also disappointing (121). Although suppressing the VEGF pathway indeed decreases vascular density, rapid revascularization occurs within 2 weeks as shown in a neoadjuvant window-of-opportunity bevacizumab study (5, 39, 119). This is likely mediated through induction of hypoxia by the antiangiogenic therapy, resulting in compensatory upregulation of both VEGF and VEGF-independent angiogenesis pathways (119, 122). Proposed resistance mechanisms include vascular mimicry, enhancement of invasive potential, recruitment of

bone marrow-derived precursor endothelial cells, and promotion of alternative proangiogenic pathways (5, 39, 42, 123), which are of interest as potential therapeutic targets in breast cancer.

Hypoxia created by VEGF pathway inhibitors correlates with upregulation of the *MET* oncogene, which promotes invasive behavior and is an adverse prognostic factor in breast cancer (42, 123, 124). Cabozantinib (XL-184) is a potent oral inhibitor of MET and VEGFR2, and phase II trials showed mixed clinical benefit rates (0%–34%) in metastatic TNBC (125, 126).

In TNBC xenografts, dual FGF/VEGF targeting with or without paclitaxel chemotherapy showed synergistic effects in reducing vessel number and growth (127, 128). In a phase II trial of the dual FGF/VEGF inhibitor brivanib in solid tumors, responses were seen in breast cancer patients; however, this cohort was terminated early (129). Nintedanib, an inhibitor of VEGFR, PDGFR, and FGF receptors (FGFRs) that is approved for non-small cell lung cancer, showed preclinical activity in combination with paclitaxel in breast cancer xenografts and is being tested in breast cancer patients (130, 131). Interestingly, FGFR signaling also appears to mediate resistance to CDK4/6 inhibitors in breast cancer (132).

Trebananib (AMG386) is an ANGPT antagonist peptide-Fc fusion protein that selectively binds ANGPT1 and ANGPT2 (133). However, a phase II clinical trial in metastatic breast cancer patients indicated no evidence of benefit when combining AMG386 and paclitaxel with bevacizumab (133).

Src kinase is required for VEGF-induced proliferation of vascular cells, for vascular permeability, and for tumor cell extravasation in preclinical models (134). In phase II breast cancer studies, circulating VEGFR increased during exposure to the Src inhibitor dasatinib, implying that combination of VEGF and Src inhibitors may also be of interest (134).

Inhibition of angiogenesis may result in selection of cells that can use existing vasculature, known as co-option, a growth pattern observed in breast cancer liver metastases (135). In patients with colorectal cancer liver metastases, co-option was associated with poor response to bevacizumab (136). Inhibitors of key players in co-option such as the actin-related protein 2/3 complex (Arp2/3), also expressed in breast cancer liver metastases, enhanced the efficacy of angiogenesis inhibitors in preclinical models of liver metastases (136).

Pharmaceutical targeting of metabolism in breast cancer. In preclinical breast cancer models, agents that directly interfere with high glucose uptake (e.g., the glucose analog 2-deoxyglucose) or decrease glycolysis (e.g., the PDK inhibitor dichloroacetate) reduced proliferation, inhibited HIF-1 α , and sensitized cells to chemotherapy and mitochondrial inhibitors (137–139). Although phase I clinical cancer trials have included some breast cancer patients, toxicity has been a problem and no clear efficacy signals have emerged (140).

Lactate dehydrogenase (LDH) is a key enzyme for the interconversion of pyruvate and lactate. Although its complex biochemistry and multiple isoenzymes have made it hard to “drug” (141), several molecules are of interest for further development in cancer, including the old anticonvulsant stiripentol, which inhibits LDHA in vivo (142). Other ways to target lactate metabolism include blocking its transmembrane transport by inhibiting MCT1 and MCT4 (143–145). Inhibition of MCT1 in breast cancer was

effective preclinically; however, the main mechanism appeared to be reduced pyruvate export rather than altered lactate transport or reduced glycolytic flux (146). The major immunosuppressive effect of extracellular lactate (147, 148) makes combinations of inhibitors of lactate transport with immune checkpoint inhibition of interest, especially in TNBC, in which checkpoint inhibition has proven effectiveness when combined with chemotherapy. Indeed, MCT1 blockade with AZD3965 increases immune cell infiltration in tumors, and inhibiting CA9 enhances immune responses to PD-L1 inhibition (149, 150). AZD3965 and the CA9 inhibitor SLC-0111 are currently in phase I cancer trials.

Dependence of breast cancer cells on glutamine is increased not only in hypoxia but also in estrogen-independent and anti-estrogen treatment-resistant subtypes (151). Preclinically, pharmacological targeting of HIF-regulated amino acid importers, for instance by the SLC1A5 inhibitors benzylserine or V-9302, blocks breast cancer cell growth and is associated with decreased mTOR signaling and increased ROS levels and autophagy (69, 71, 152, 153). Inhibition of GLS by CB-839 also inhibits growth of TNBC cells but not ER α breast cancer cells, which rely on GLS2 instead (154). Combining CB-839 with the mTOR inhibitor everolimus, however, does inhibit growth of endocrine-resistant breast cancer xenografts (151, 155). This is of interest since mTOR inhibition is already being used clinically in combination with hormonal therapy in ER α patients to prevent endocrine resistance. CB-839 is now being evaluated in early clinical (breast) cancer trials.

Regarding cancer cell lipid metabolism, blocking FA synthesis has received the most attention, and, in vitro, inhibiting FASN reduced proliferation and induced apoptosis (77). TVB-2640 is a specific FASN inhibitor that has now proceeded into a phase II breast cancer trial. Interestingly, proton pump inhibitors such as omeprazole also inhibit FASN (156). The proton pump inhibitor omeprazole improved survival in metastatic breast cancer patients receiving chemotherapy, making repurposing of this FDA-approved class of drugs of interest, and further clinical evaluation is ongoing (157).

Targeting of components in the glycolytic pathway and vascular normalization induced by antiangiogenic therapy increase dependence of cancer cells on mitochondrial metabolism. Metformin, an AMPK activator that is a cornerstone in the treatment of type 2 diabetes, inhibits mitochondrial complex 1. More recently, it has also been shown to inhibit growth differentiation factor 15 (GDF15), a HIF-1 target (158). In the preclinical setting, metformin increased internalization of caveolin-1/T-DM1 and sensitivity to T-DM1 treatment through suppression of the HIF-responsive Akt/MAPK pathway (159). Metformin is one of the main metabolically targeted drugs currently under investigation in breast cancer with (combination) trials ongoing in the setting of prevention and maintenance (160). However, so far no benefit of metformin has been demonstrated in randomized trials, which may be related to compensatory increases in glucose uptake and transcription of many genes involved in mitochondrial metabolism that occur already within 1–2 weeks of treatment (161).

In a phase 0/I randomized trial in HER2, treatment-naïve primary breast cancer patients, single-dose bevacizumab treatment was followed by randomization to treatment with the mitochondrial inhibitor ME-344 or placebo. In paired pre- and

post-treatment biopsies, reduced proliferation was demonstrated in ME-344-treated patients, especially in the subgroup that had vascular normalization measured using ^{18}F -FDG PET (162). This illustrates the type of trial design and smart drug combinations that will be essential for further therapeutic development.

Several agents that target ROS are being studied alone or in combination, including decylubiquinone, an FDA-approved coenzyme Q_{10} analog that inhibits angiogenesis in breast cancer cells through a ROS-dependent mechanism (163).

Nonpharmaceutical targeting of metabolism in breast cancer. Nonpharmaceutical interventions that take advantage of the metabolic differences between cancer cells and normal cells, many mediated by HIF-dependent pathways, are also of interest. Exercise is of increasing importance in breast cancer care and is associated with decreased tumor growth and improved patient mental well-being and survival. Reduction of ROS is one of the multiple hypothesized underlying mechanisms (164). Of specific dietary interventions that have been proposed to have anticancer effects, ketogenic diets and fasting have received the most attention (165, 166).

Ketogenic diets are based on the premise that cancer cells are more dependent on glucose and have defective mitochondrial metabolism compared with normal cells. These diets are composed of high fat, moderate protein, and low carbohydrate content, resulting in increased fat metabolism. FAs are oxidized in the liver to acetyl-CoA, and any excess is converted into ketone bodies, mainly β -hydroxybutyrate. Normal tissues, in contrast to cancers, have the ability to use ketones as a source of energy, thus making these diets more detrimental to cancer cells. Many cancer trials have been initiated to investigate the ketogenic diet and have shown feasibility and reduced central obesity and insulin levels but no clear anticancer efficacy (167, 168). It is now well recognized that mitochondria continue to be functional in cancers, reducing the likelihood of large effect sizes. Furthermore, effects may be compensated by utilization of extracellular β -hydroxybutyrate by breast cancers for acetyl-CoA production (169).

Fasting decreases glucose, insulin, and IGF-1 levels while increasing FA breakdown and production of ketones, similar to the ketogenic diet (166, 170). Reducing IGF-1 reduces Akt signaling, and lower glucose increases AMPK activity. In 13 breast cancer patients, short-term fasting appeared to reduce hematological toxicity of neoadjuvant chemotherapy, possibly through faster recovery of DNA damage in PBMCs (171). Nevertheless,

ketogenic and fasting diets are extremely challenging to adhere to, especially for cancer patients in whom malnutrition is detrimental to quality of life, response to therapy, and survival. Thus, although many behavioral modifications have a promising metabolic rationale exploiting the Warburg effect and ROS, strong and mechanistic proof for direct anticancer efficacy from translational studies is warranted.

Concluding remarks

HIFs and downstream angiogenic and metabolic alterations play a major role in breast cancer aggressiveness, progression, and therapy resistance but have proven to be notoriously difficult targets in the clinic. Novel druggable targets in HIF upstream regulatory pathways and downstream angiogenic and metabolic pathways are increasingly being identified. Continuous technological developments in (noninvasive) measurement of tumor glucose uptake, hypoxia, and vasculature now enable real-time in vivo monitoring of treatment-induced alterations. Approaches to clinically study the fate of metabolites are important for stratification and for understanding responses and escape mechanisms, and novel metabolic tools such as ^{18}F -glutamine PET/CT and ^{13}C -metabolite flux tracing have been developed for clinical use or are in development, e.g., ^{18}F -labeled MCT inhibitors (161, 172–174). Smart incorporation of these tools into trials at baseline and interim time points can aid in successful translation of proposed antiangiogenic and metabolically targeted therapies to the clinic. Since the narrow therapeutic window and rapid emergence of escape mechanisms have posed major hurdles to monotherapies targeting these pathways, combination of novel antiangiogenic and metabolic drugs with existing therapies and nonpharmaceutical interventions seems most promising.

Acknowledgments

ALH has research funding from the Breast Cancer Research Foundation, Cancer Research UK (grant number A18974), and the Kennington Cancer Fund. MJ has research funding from the Dutch Cancer Society (Young Investigator Grant 10913/2017-1).

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